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FACULDADE DE MEDICINA VETERINÁRIA

MODULATING CHROMATIN STRUCTURE AND GENE EXPRESSION DURING AFRICAN SWINE FEVER VIRUS INFECTION – NEW STRATEGIES FOR AN EFFICIENT VACCINE RATIONAL DESIGN

Gonçalo Daniel dos Santos Frouco

Orientador: Professor Doutor Fernando António da Costa Ferreira
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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Sanidade Animal

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Modulating chromatin structure and gene expression during African Swine Fever Virus infection – New strategies for an efficient vaccine rational design

ABSTRACT

African swine fever virus (ASFV) is a nucleo-cytoplasmic large DNA virus which infects all members of the family Suidae, causing a fatal disease of domestic swine and wild boar. Since no effective vaccine or treatment is available, ASF is considered a global threat for pig husbandry. The ASFV genome encodes among others, enzymes required for virion assembly, genome transcription and replication, including a putative histone-like protein, pA104R. In bacteria, these proteins perform topological modification of the chromosome (twisting, bending and folding), playing important structural and regulatory functions. Since ASFV has a large genome, a viral histone-like protein may be important for packaging its genome within the virion particle and/or for viral replication and transcriptional events. In this study, the ASFV-pA104R activity was characterized and its DNA-binding activities were evaluated. pA104R binds both to ssDNA and dsDNA, although having higher affinity to ds-DNA, over a wide range of temperatures, pH values, and salt concentrations and in an ATP-independent manner, with an estimated binding site size of about 14 to 16 nucleotides. The arginine residue located in pA104R's DNA-binding domain, at position 69, also revealed to be important for an efficient DNA-binding. Additionally, since pA104R together with the viral type II topoisomerase, pP1192R, displayed DNA-supercoiling activity, a synergistic effect between these viral is proposed. The expression of pA104R was observed in the late phase of infection in infected cells with the Vero-adapted ASFV isolate Ba71V, co-localizing with cell nucleus and viral factories. siRNA experiments showed that the knockdown of A104R induce a reduction of viral progeny, copy numbers of viral genomes and transcription of a late viral gene, revealing that pA104R plays a critical role in viral DNA replication and gene expression. Results obtained on these studies prompted us to pursue the objective to generate a defective infectious single cycle (DISC) ASFV lacking the A104R gene. Recombinant virus was successfully obtained, however the complementary cell line previously developed did not support its replication. The antiviral activity of four HDACi against ASFV was also evaluated in this study. The results showing the abrogation of viral replication by NaPB open new insights on its use as an antiviral strategy to control ASFV spreading. Overall our data strongly support that pA104R plays an important role on ASFV replication opening a new window for the design of ASF control measures through the development of efficient and safe vaccines and antivirals.

Keywords: African swine fever virus, ASFV, histone-like protein pA104R, vaccine, defective infectious singe cycle viral particles, antivirals.

Modulação da estrutura cromatínica e da expressão génica durante a infeção do Vírus da Peste Suína Africana – novas estratégias para o desenvolvimento de uma vacina eficaz

RESUMO

O vírus da peste suína africana (VPSA) é um vírus de DNA nucleo-citoplasmático que infeta todos os membros da família *Suidae*, causando uma doença com elevada mortalidade em suínos domésticos e nos javalis. Atualmente não existe uma vacina ou tratamento eficaz, tornando a peste suína africana (PSA) uma ameaça para a suinicultura mundial. O genoma do VPSA codifica aproximadamente 150 proteínas, algumas delas bem caracterizadas, estando envolvidas na transcrição, replicação ou na montagem do virião. No entanto, e apesar de todos os esforços realizados nas últimas décadas, a função biológica de numerosas proteínas virais não é ainda conhecida. Esta lacuna aliada à necessidade de um melhor entendimento sobre a biologia do VPSA e as suas interações com o hospedeiro têm contribuído em grande parto para a dificuldade no desenvolvimento de uma vacina eficaz contra PSA.

Por homologia de sequências proteicas, o genoma do VPSA codifica para uma proteína tipo histona (pA104R). Nas bactérias, estas proteínas são responsáveis por modular a topologia do DNA (torção, flexão e dobramento), desempenhando assim importantes funções estruturais e controlando a expressão de diferentes genes. O facto do genoma do VPSA codificar entre outras uma proteína viral semelhante a histonas bacterianas, reveste-se assim de enorme relevância pelo papel que que estas proteínas possam desempenhar na compactação do genoma na partícula viral e/ou para a sua replicação e transcrição. Neste contexto, este estudo pretendeu caracterizar o papel da VPSA-pA104R na replicação viral, tendo como objetivo contribuir para o conhecimento da biologia deste vírus e para averiguar se o gene A104R será um bom candidato para desenvolver uma vacina DISC (do inglês “defective infectious single cycle). Além disso, diferentes inibidores das histonas deacetilases (HDACs) foram testados como potenciais antivirais, eventualmente úteis no controlo da PSA

Os principais objetivos deste trabalho foram assim os seguintes: (1) estudar VPSA-pA104R, através da clonagem, expressão, purificação e caracterização de sua atividade *in vitro*; (2) Compreender a relevância funcional de dois resíduos conservados de pA104R; (3) Avaliar os níveis de mRNA e proteína, bem como a localização intracelular de pA104R em células infetadas com VPSA, em diferentes tempos de infeção; (4) Desenvolver uma estratégia que permita a deleção da ORF A104R do genoma do VPSA e a obtenção de uma vacina DISC; (5) avaliar os níveis de acetilação das histonas das células infetadas para melhor compreensão do mecanismo de modulação dos mecanismos epigenéticos do hospedeiro pelo VPSA; (6) Avaliar o efeito dos inibidores das HDACs na infeção pelo VPSA.

Neste estudo, a VPSA-pA104R foi expressa num sistema procariota baseado em *Escherichia coli*. Após a sua purificação, a sua atividade foi caracterizada através de ensaios EMSA (do inglês “electrophoretic mobility shift assay”) e concluiu-se que esta proteína viral se liga tanto a DNA de cadeia simples como dupla, embora tenha maior afinidade para o de cadeia dupla, e estimou-se que o local de ligação seja cerca de 14 a 16 nucleótidos. Esta ligação ao DNA continua presente em variadas condições de temperaturas, pH e concentrações de sal e é independente de ATP. A perda de atividade da proteína mutada pontualmente no resíduo de arginina localizado na posição 69, revelou que este resíduo é importante para uma ligação eficaz ao DNA. Além disto, foi possível concluir que a carga positiva deste aminoácido é determinante para a capacidade de ligação do pA104R ao DNA. Adicionalmente, uma vez que se observou atividade de superenrolamento de DNA quando a pA104R e uma topoisomerase tipo II viral, pP1192R, foram adicionados a DNA plasmídeo relaxado, este trabalho suporta que o VPSA codifica de facto para proteínas necessárias a compactação do seu genoma e ainda é proposto a existência de um efeito sinérgico entre as duas proteínas virais acima descritas.

Como o objetivo de melhor compreender a importância da pA104R na infeção do VPSA, avaliou-se a dinâmica da sua expressão e a sua localização intracelular. A expressão de pA104R foi observada na fase tardia da infeção em células Vero infetadas com o isolado viral Ba71V, co-localizando com núcleo da célula e fábricas virais citoplasmáticas. Em relação à dinâmica de transcrição do gene A104R, apesar de ser típica de um gene tardio, foi possível detetar transcritos a partir das 2 horas pós-infeção (hpi). As experiências usando siRNA (do inglês “small interference RNA”) contra os transcritos do gene A104R, mostraram que a redução dos níveis de RNA deste gene induzem uma redução da progenia viral, do número de cópias de genomas virais e da transcrição de um gene viral tardio (B646L), revelando que o pA104R desempenha um papel crítico na replicação do DNA viral e na expressão de genes virais.

Atualmente, as únicas medidas de controlo do VPSA são baseadas na deteção precoce da doença e na rápida aplicação de medidas biossanitárias como o abate de animais infetados, controlo do movimento de animais e a vigilância. As tentativas falhadas até agora em obter uma vacina inativada ou atenuada permitem que novas estratégias, como as vacinas DISC ganhem espaço na investigação do VPSA como uma revigorante estratégia para controlar o VPSA. Os resultados obtidos neste estudo, anteriormente descritos, suportam a ideia que um mutante de deleção no gene A104R replicará o seu genoma nas células hospedeiras, mas não poderá compacta-lo dentro do virião, resultando num virião não-infecioso "vazio" que será incapaz de iniciar um segundo ciclo de infeção. Assim a infeção com este vírus mutante será capaz de estimular o sistema imunitário do hospedeiro, mas ao mesmo tempo será seguro, não produzindo progenia infeciosa. Assim outro objetivo deste trabalho foi então obter um vírus DISC deletado no gene A104R. Para isto, o vírus recombinante foi obtido por

recombinação homóloga e uma linha Vero complementar, que expressa a pA104R, foi desenvolvida. Embora, o vírus recombinante tenha sido obtido com sucesso, a linha celular complementar desenvolvida não suporta a sua replicação e, como tal, a seleção e propagação do vírus recombinante não foi possível. Os baixos níveis de expressão de pA104R destas células quando comparados com os de células infetadas poderão explicar esta não complementação.

Com base em estudos anteriores que mostram que VPSA regula o estado epigenético da célula hospedeira, o grau de acetilação das histonas de células infetadas foi avaliado e a atividade antiviral de quatro inibidores das HDACs (NaPB, VPA, TSA e SAHA) contra a infeção pelo VPSA também foi testada neste estudo. O VPSA induz uma hipoacetilação dos resíduos de lisina 9 e 14 da histona H3 (H3K9K14). Esta modificação epigenética corrobora outras reportadas noutros estudos e todas elas estão classicamente correlacionada com o silenciamento de genes em células eucarióticas e pode indicar que o VPSA subverte diferentes mecanismos celulares, controlando o acesso da maquinaria de transcrição aos genes hospedeiros. Adicionalmente, um dos inibidores das HDACs testados, o NaPB, reverte este estado de hipoacetilação da histona e inibe a replicação do VPSA, interferindo com a expressão de gene virais tardios.

Os resultados obtidos neste estudo sugerem fortemente que a pA104R participa da modulação da topologia do DNA viral, estando envolvida na replicação, transcrição e/ ou compactação do DNA viral. Com o objetivo de desenvolver uma vacina DISC, o gene A104R poderá assim constituir um bom alvo a deletar. Esta nova estratégia poderá ser uma alternativa às tentativas até agora falhadas de obter uma vacina contra a PSA. Contudo, antes de uma vacina DISC ser realidade, um esforço científico no desenvolvimento de uma linha celular complementar será imperativo. Os resultados obtidos sugerem ainda que as HDACs celulares estão envolvidas no estabelecimento de infeção pelo VPSA e revelaram que o NaPB pode ser usado como uma estratégia antiviral adicional para controlar a propagação de vírus nas áreas de surto.

Palavras-chave: Vírus da peste suína africana, VPSA, proteína tipo-histona pA104R, vacinas, partículas virais “defective infectious singe cycle”, antivirais.

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ADP	adenosine 5′-diphosphate
AraC	cytosine arabinoside
ASF	African swine fever
ASFV	African swine fever virus
ATP	adenosine 5′-triphosphate
bp	base-pair
BSA	bovine serum albumin
CbpA	curved DNA binding protein A
CbpB	curved DNA binding protein B
cDNA	complementary DNA
CPE	cytopathic effect
DAPI	4′,6-diamidino-2-phenylindole
DISC	defective infectious single cycle
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
DPS	DNA binding protein from starved cells
EBV	Epstein-Barr virus
ECACC	European Cell Culture Collection
EDTA	ethylenediaminetetraacetic acid
EGCG	(-)-epigallocatechin gallate
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
FBS	fetal bovine serum
Fis	factor for inversion stimulation
FITC	fluorescein isothiocyanate
GUS	beta-glucuronidase
HBV	Hepatitis B virus
hpi	hour(s) post-infection
HAT	histone acetyltransferases
HATi	histone acetyltransferases inhibitors
HDAC	histone deacetylases
HDACi	histone deacetylases inhibitors
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H-NS	histone-like nucleoid structuring protein
HP1	Heterochromatin Protein 1
HRP	horseradish peroxidase
HSV	Herpes simplex virus
HU	histone-like protein from E. coli strain U93
H3K4me2	histone H3 dimethylated at lysine 4
H3K9K14	histone H3 lysine 9 and 14
H3K9K14Ac	histone H3 lysine 9 and 14 acetylation
H3K9me	histone H3 methylated at lysine 9
H3K9me3	histone H3 trimethylated at lysine 9
IHF	integration host factor
IPTG	isopropyl-β-D-1-thiogalactopyranoside
kbp	kilo base-pair
kDa	kilo Dalton
Lrp	leucine responsive protein
MOI	multiplicity of infection
mRNA	messenger RNA

MTOC	microtubule organization center
ncRNA	noncoding RNA
NAP	nucleo-associate protein
NaPB	sodium phenylbutyrate
NCLDV	nucleo-cytoplasmic large DNA virus
nt	nucleotides
OIE	Office International des Épizooties – World Animal Organization
ORF	open reading frame
PIPES	piperazineN,N'-bis(2-ethanesulfonic acid)
PBS	phosphate-buffered saline
PBST	PBS supplemented with 0.1% Tween-20
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PTM	posttranslational modifications
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
SAHA	vorinostat
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SFM	scanning force microscopy
SMC	structural maintenance of chromossomes
StpA	suppressor of the Td' phenotype
SV40	Simian virus 40
TBE	Tris-borate-EDTA
TCID50	tissue culture infectious dose 50%
TSA	trichostatin A
Tx	Triton X-100
VPA	valproic acid
v/v	volume per volume
wt	wild-type
w/v	weight per volume
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid

CHAPTER I

Introduction

1. African Swine Fever

1.1. History and distribution

African swine fever (ASF) is nowadays considered one of the most threatening diseases of pig husbandry. First described by Montgomery (1921) as an acute and fatal haemorrhagic fever affecting domestic pigs in Kenya, ASF remains endemic in most sub-Saharan African countries. The first spread of ASF outside Africa was reported in Portugal in 1957, as result of contaminated waste from airline flights which were provided to pigs near the Lisbon airport. Despite the prompt control and eradication of the disease, a further outbreak occurred in 1960 in Lisbon (Manso Ribeiro & Azevedo, 1961), with a subsequent spread to many areas of the Iberian Peninsula, where ASF remained endemic until 1995 (Sánchez-Vizcaíno, Mur, & Martínez-López, 2012). During the 1970s and 1980s ASF affected other countries in Europe, such as France, Italy, Malta, Belgium and Netherlands, as well as several Caribbean countries – Cuba, Dominican Republic and Haiti – and Brazil (Costard et al., 2009).

As consequence of control measures adopted, ASF was eradicated from those territories, with the exception of Sardinia, in Italy, where the disease persists up to date. However, in the African continent, the incidence of ASF increased during the 1990s and 2000s decade in endemic countries and entered in other regions not typically affected by the disease [Côte d'Ivoire (1996), Nigeria (1997), Togo (1997), Madagascar (1998), Ghana (1999), Burkina Faso (2003), Mauritius (2007) and Chad (2010)] (Sánchez-Vizcaíno et al., 2012). The increase of African swine fever virus (ASFV) circulating in Africa, together with the number of infected animals and contaminated products may contributed to the re-emergency of the disease in the European Continent via Georgia, in 2007 (Sánchez-Vizcaíno et al., 2012). The molecular analysis of the ASFV found in Georgia outbreak revealed a close relationship to virus circulating in Mozambique, Zambia and Madagascar (Rowlands et al., 2008), confirming the origin from South Eastern Africa or Madagascar of the disease in the Caucasus region. Since the first clinical cases were disclosed in the area surrounding the port of Poti (Black Sea), the virus was probably introduced through improperly disposed waste products from international ships carrying contaminated pork products, which were then used to feed pigs (Beltran-Alcrudo, Lubroth, Depner, & De La Rocque, 2008). From this initial outbreak the virus spread very quickly to other regions of Georgia, Armenia, Azerbaijan and Russia Federation, increasing the risk of introduction of ASF into the European Union (Sánchez-Vizcaíno, Mur, & Martínez-López, 2013; Gogin, Gerasimov, Malogolovkin, & Kolbasov, 2013). Due to the abundance of backyard pig units and areas of interaction between free-ranging pigs and wild boar, the illegal movement of infected animals and products, the lack of biosecurity measures and poor implementation of control measures, ASF is maintained in these regions and continued to spread into Ukraine (2012) and Belarus (2013) (Gallardo et al., 2015).

The first introduction of ASF in the EU was registered in Lithuania (2014), and rapidly further notifications were occurred in Poland, Latvia, Estonia, Czech Republic, Moldova and Romania (Gallardo et al., 2015; OIE, 2017). The recently re-introduction of ASF into EU area emphasizes the actual threat of this disease to the global pig husbandry.

1.2. Epidemiology of African swine fever

1.2.1. African swine fever hosts

1.2.1.1. Domestic pigs

The clinical and pathological signs of ASF in domestic pigs vary considerably depending on the virulence of the ASFV strain and host factors like its immunological status (Costard, Mur, Lubroth, Sanchez-Vizcaino, & Pfeiffer, 2013). Acute infections with highly virulent virus strains are characterized by a massive apoptosis of lymphocyte and a haemorrhagic fever with an impairment of haemostasis and immune functions, and are associated with high morbidity and mortality rates (Blome, Gabriel, & Beer, 2013; Sánchez-Vizcaíno, Mur, Gomez-Villamandos, & Carrasco, 2015). Subclinically infected, chronically infected and recovered pigs are likely to play an important role in the ASF epidemiology, being responsible for the persistence of the disease in endemic areas, sporadic outbreaks and introduction into disease-free zones (Allaway, Chinombo, Edelsten, Hutchings, & Sumption, 1995; Gallardo et al., 2015).

1.2.1.2. Wild suids

The warthog (*Phacochoerus africanus*) is considered the original vertebrate host and the most important reservoir of ASFV in Africa (Jori & Bastos, 2009; Costard et al., 2013). Infected adult warthogs are asymptomatic carriers of the disease, however viral replication and viremia occur in young animals which are infected by soft ticks carrying the virus, present in their burrows (Thomson, Gainaru, & Dellen, 1980).

The role of bushpigs (*Potamochoerus larvatus*), red river hogs (*Potamochoerus porcus*), and giant forest hogs (*Hylochoerus meinertzhageni*) in the ASF epidemiology remains not fully elucidated (Costard et al., 2009; Jori & Bastos, 2009). Although the bushpigs are susceptible to infection and develop sufficient levels of viremia to infect pigs and soft ticks, they do not show any clinical signs (Anderson, Hutchings, Mukarati, & Wilkinson, 1998; Oura, Powell, Anderson, & Parkhouse, 1998). Since these animals rarely interact to domestic pigs and soft ticks, due to the low densities of their population, their nocturnal habits and non-use of burrows, bushpigs are not considered important reservoirs of ASFV (Costard et al., 2013).

In Europe, wild boar (*Sus scrofa*) and feral pigs are highly susceptible to both natural and experimental ASFV infection (McVicar, Mebus, Becker, Belden, & Gibbs, 1981; Jori & Bastos, 2009) and, similarly to domestic pigs, the clinical signs of disease may vary considerably from peracute death to unapparent courses (Blome et al., 2013). Although, some studies revealed that the virus cannot persist in wild boar populations for long periods of time without the re-

infection by contact with infected domestic pigs and contaminated products (Laddomada et al., 1994; Mur, Martínez-López, & Sánchez-Vizcaíno, 2012), these animals facilitate the spread of the disease to free-range pigs (Costard et al., 2013; Gallardo et al., 2015).

1.2.1.3. Soft ticks

ASFV infected *Ornithodoros* spp. ticks, may harbor high levels of virus and a persistent infection (Boinas, Wilson, Hutchings, Martins, & Dixon, 2011; Burrage, 2013). These infected soft ticks may play an important role on ASF epidemiology, acting as vectors of the disease both for domestic and wild pigs and also being a long-term reservoir of the disease, as was suggested in Portugal when, in 1999, ASF re-emerged on a farm that had been affected previously and where infected ticks were found (Beloin et al., 2006; Costard et al., 2009; Boinas et al., 2011).

1.2.2. Transmission

ASFV is transmitted by direct contact between infected and non-infected pigs (through blood, secretions and excretions), by consumption of meat and pork products from infected pigs, by bites of infected soft ticks, and through materials contaminated by blood, faeces, urine or saliva from infected animals (Guinat et al., 2016; Penrith & Vosloo, 2009). Infection of susceptible pigs normally occurs either orally or nasally, via tonsils or dorsal pharyngeal mucosa to the mandibular or retropharyngeal lymph nodes, from where the virus spread through viremia (Sánchez-Vizcaíno et al., 2009).

The virus is very resistant to inactivation and can persist and remain viable in the environment for long periods, thus enabling the transmission either by direct contact or via fomites (e.g. contaminated clothes and footwear, vehicles, equipment, bedding) for several weeks and turning the ASF control difficult (Costard et al., 2013; Davies et al., 2015).

In the sylvatic transmission cycle of ASF in Africa, which involves warthogs and ticks of the *Ornithodoros moubata* complex, there is not horizontal or vertical transmission between warthogs, thus the maintenance of infection is exclusively dependent on infected ticks (Jori & Bastos, 2009). This vector transmits the virus to young suckling warthogs, which have a short period of viremia, allowing the transmission to uninfected ticks during blood meals (Thomson et al., 1980). Since the transmission by direct contact seems improbable in this scenario, the transmission from sylvatic to domestic pigs, is attributed to the argasid ticks (Jori & Bastos, 2009).

Although, several studies indicate that *Ornithodoros* ticks can maintain ASFV infection for several months or even years and were responsible for ASF outbreaks in Iberian Peninsula (Basto et al., 2006; Boinas et al., 2011; Pérez-Sánchez, Astigarraga, Oleaga-Pérez, & Encinas-Grandes, 1994; Sanchez-Botija, 1963), their involvement in the current spread of the disease in the Caucasus, Russia and Eastern Europe is unlikely (Jori & Bastos, 2009; Guinat

et al., 2016). In this scenario, infected wild boars are an effective transmission route to domestic pigs by among others, direct contact and through ingestion of infected carcasses, being currently the greatest concern in the control of ASF nowadays in EU (Gabriel et al., 2011; Gallardo et al., 2015).

1.3. Strategies to control African swine fever

1.3.1. Sanitary/ biosecurity control measures

ASF is a viral swine disease, entailing high mortality rates and great sanitary and socioeconomic impact in affected countries, and it is listed as a notifiable disease by the World Organization for Animal Health (OIE) (Costard et al., 2013). Allied to its complex epidemiology and transmission and to a marked resistance of the ASFV in contaminated material and animal products (Sánchez-Vizcaíno et al., 2012), ASF control and eradication is complex and difficult due to the absence of a vaccine or an effective treatment against this disease. Thus, its control is based on the early detection and on an efficient laboratory diagnosis of the disease and the implementation of sanitary and biosecurity control measures, namely animal slaughter, movement restrictions, notification and surveillance (Wieland, Dhollander, Salman, & Koenen, 2011). The clinical diagnosis of the ASF is often complicated due to the similarity to other swine diseases and to the wide range of clinical forms of the disease, ranging from highly lethal to subclinical (Fig. 1). Highly virulent strains are usually responsible for the peracute and acute forms of the disease, characterised by high mortality rates after 1-4 and 4-9 days post-infection, respectively. These acute forms are characterised by a febrile syndrome with erythema and cyanosis of the skin, functional failures of internal organs, vomiting and haemorrhagic diarrhoea. In subacute forms of the disease, a persistent or fluctuating fever lasts for up to 20 days and the mortality rates are in the range of 30-70%, while in chronic forms clinical signs and lesions are not specific (delayed growth, emaciation, skin ulcers, arthritis, pneumonia, abortion) and could persist for several months (Blome et al., 2013; Gallardo et al., 2015; Sánchez-Vizcaíno et al., 2015).

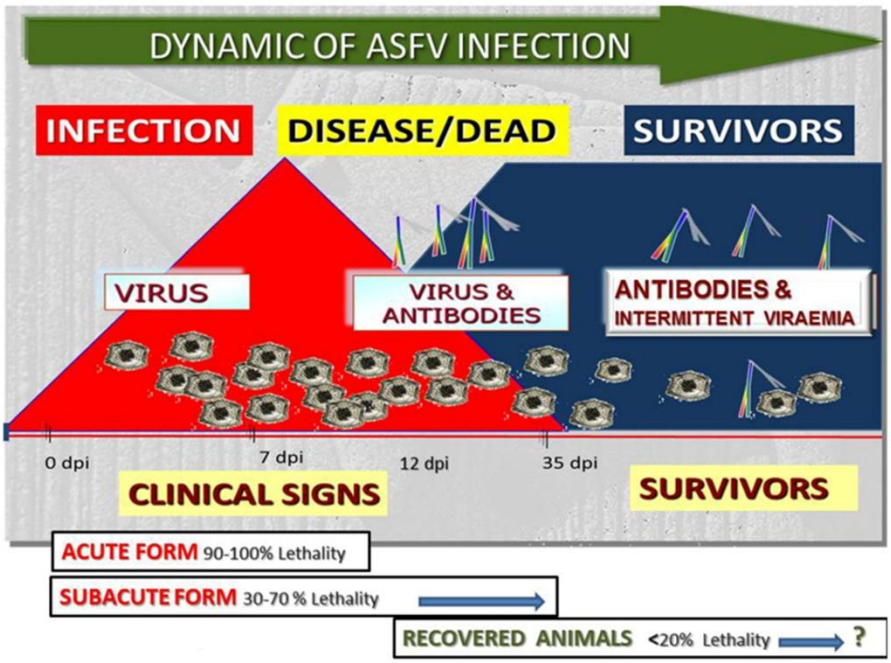

Figure 1. Dynamic of ASF infection.
The picture summarizes the ASFV appearance in blood and antibodies after infection. The lethality of the different forms of the clinical disease, which ranges from acute to a subacute, as well as from recovered animals are also shown. Figure and legend were adapted from Gallardo et al. (2015).

1.3.2. Development of ASF vaccine

Despite all the efforts to develop an ASF vaccine, no vaccine is currently available to prevent and control the global spread of ASF. The complexity of the virus, with genes involved in the evasion of the host immune response, the variation extent of ASFV strains and the large gaps in knowledge concerning ASFV infection and immunity, contribute to the unsuccessful of all classical attempts to generate a vaccine using inactivated or live-attenuated virus (Rock, 2016). Experiments using traditional inactivated vaccines, such as inactivated infected cell extracts, supernatants of infected pig peripheral blood leukocytes, purified and inactivated virions, infected glutaraldehyde-fixed macrophages, and detergent-treated infected alveolar macrophage cell cultures, have failed to induce protection (Forman, Wardley, & Wilkinson, 1982; Mebus, 1988; Stone & Hess, 1967). Recently, Blome, Gabriel, & Beer (2014) also observed that adjuvants do not enhance the efficacy of these classical vaccines.

Nevertheless, studies showed that a solid immune response is induced in surviving pigs (Boinas, Hutchings, Dixon, & Wilkinson, 2004; Hamdy & Dardiri, 1984; Mebus & Dardiri, 1980) and animals infected with ASFV attenuated or moderately virulent develop protection against homologous virus (King et al., 2011; Lacasta et al., 2015; Leitão et al., 2001; Lewis et al., 2000; O'Donnell et al., 2015; Reis et al., 2016), suggesting that an effective vaccine against ASFV infection could indeed be feasible.

Unfortunately, significant safety issues (residual pathogenicity and potential for long-term viral persistence) were associated until now with live-attenuated vaccines (King et al., 2011; Lacasta et al., 2015; Lewis et al., 2000; Manso Ribeiro & Azevedo, 1961). Concerning ASF

subunit vaccines, although several ASFV proteins have been associated with protection (Lokhandwala et al., 2016; Lokhandwala et al., 2017), the available data shows that these vaccines are insufficient for the induction of a protective immunity in pigs (Argilaguet et al., 2013; Neilan et al., 2004; Rock, 2016).

1.3.3. Development of antiviral agents

In the absence of an effective vaccine against ASF, several antiviral agents have been identified. Although, some compounds have demonstrated strong anti-ASFV activity in vitro (e.g. rifamycin, fluoroquinolones, lauryl-gallate, methyl-b-Cyclodextrin, amiloride, sulfated polysaccharides, lysosomotropic agents, valproic acid, apigenin) (Cuesta-Geijo et al., 2012; Freitas, Frouco, Martins, Leitão, & Ferreira, 2016; García-Villalón & Gil-Fernández, 1991; Hakobyan et al., 2016; Hernaez & Alonso, 2010; Hurtado et al., 2008; Mottola et al., 2013; Salas, Kuznar, & Viñuela, 1983; Sánchez et al., 2012), *in vivo* studies are mandatory to reveal if they will be potential tools for the treatment of ASFV infection.

1.4. Classification and morphology of African swine fever virus

1.4.1. Aetiology

African swine fever is caused by African swine fever virus, a large, enveloped virus with an icosahedral morphology and a double-stranded DNA genome that ranges in length between isolates about 170 to 193 kbp (Tulman, Delhon, Ku, & Rock, 2009). ASFV encodes for between 151 and 167 open reading frames (ORFs) and is the only member of the Asfarviridae family (Dixon et al., 2012), sharing general features with other members of the nucleocytoplasmic large DNA virus (NCLDV) superfamily, or as proposed recently, members of the *Megavirales* order (Colson et al., 2013).

1.4.2. Structure and composition of ASFV particles

The ASFV virion has an icosahedral morphology with an average diameter of 200 nm and is comprised by more than 50 proteins with molecular weights ranging from 10 to 150 kDa (Esteves, Marques, & Costa, 1986; Salas & Andrés, 2013). The virions also contain enzymes and factors needed for early mRNA transcription and processing (Dixon, Chapman, Netherton, & Upton, 2013; Salas, Rey-Campos, Almendral, Talavera, & Viñuela, 1986).The viral particles have a complex multi-layered structure composed by a nucleoid surrounded by a thick protein layer designated core shell, an inner lipid envelope and the capsid. The external virions also contain an external membrane, the outer envelope (Fig. 2).

The external envelope is acquired by budding from the plasma membrane of the host cells (Breese & DeBoer, 1966). The virus attachment viral protein p12, the virus homologue of cellular CD2 (pE402R) and a cellular protein p24, which is present at the plasma membrane

of host cells, have been reported to be localized into this membrane (Sanz, Garcia-Barreno, Nogal, Vinuela, & Enjuanes, 1985; Carrascosa, Saastre, Gonzalez, & Viñuela, 1993).

The viral capsid, the outermost layer of the intracellular virions, is formed by about 2000 capsomers with the appearance of hexagonal prisms and is constituted mainly by the p72, encoded by the gene B646L. Another structural protein, pB438L, and the protein pE120R, which is involved in the transport of the viral particles from the factory to the plasma membrane, are other components of the capsid (Andrés, García-Escudero, Viñuela, Salas, & Rodríguez, 2001b; Epifano, Krijnse-Locker, Salas, Salas, & Rodríguez, 2006).

The inner envelope is derived from the endoplasmic reticulum (Rouiller, Brookes, Hyatt, Windsor, & Wileman, 1998) and is composed by the membrane proteins p54, p17 and pE248R (Rodríguez, García-Escudero, Salas, & Andrés, 2004; Rodríguez, Nogal, Redrejo-Rodríguez, Bustos, & Salas, 2009; Suárez, Gutiérrez-Berzal, Andrés, Salas, & Rodríguez, 2010). This membrane surrounds the core shell, a thick protein layer which domain is mainly constituted by the processing products of polyproteins pp220 and pp62, and also by pS273R (Andrés, Alejo, Simón-Mateo, & Salas, 2001a; Andrés, Alejo, Salas, & Salas, 2002).

The nucleoid is a structure of 80 nm and contains the viral genome and nucleoproteins such as the DNA-binding protein p10 and the histone-like protein pA104R (Munoz, Freije, Salas, Vinuela, & Lopez-Otin, 1993; Borca et al., 1996). This structure also comprises the transcriptional machinery for the synthesis and modification of early RNAs (Salas & Andrés, 2013).

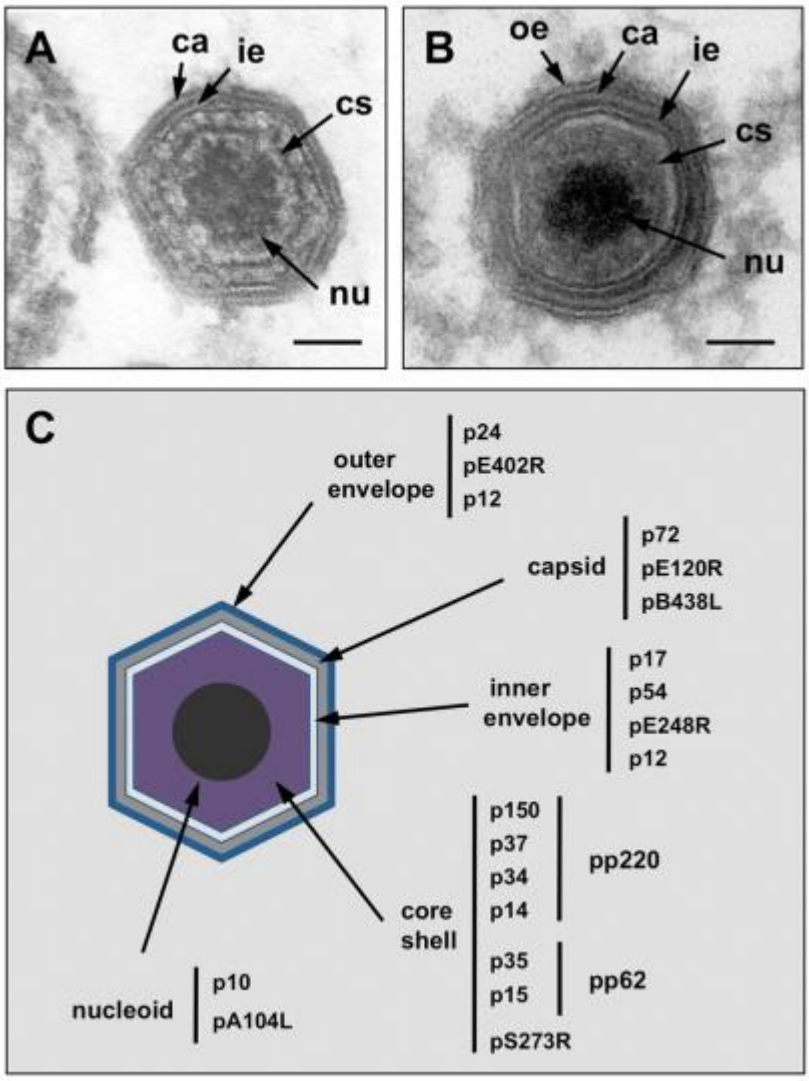

Figure 2. Structure and protein composition of ASFV particle.
(A) Electron micrograph of an intracellular full ASFV particle and (B) of an extracellular mature ASF virion. (C) Illustration of the localization of ASFV structural proteins. Outer envelope (oe), capsid (ca), inner envelope (ie), core shell (cs) and nucleoid (nu). Figure and legend were adapted from Salas and Andrés (2013).

1.5. The ASFV infectious cycle

The natural target of ASFV are cells of the mononuclear phagocytic system, and its infectious cycle normally takes between 18 to 24 h post-infection (hpi), (Costa, 1990; Muñoz-Moreno, Galindo, Cuesta-Geijo, Barrado-Gil, & Alonso, 2015). A successful infection by ASFV, as by other viruses, is a process that consists of successive steps, including virus binding, internalization and uncoating, early transcription-translation, genome replication, late protein synthesis, virus particle morphogenesis and viral egress.

1.5.1. Entry of ASFV into the host cell

Different mechanisms have been proposed for viral adsorption and entry into the host cell: phagocytosis (Basta, Gerber, Schaub, Summerfield, & McCullough, 2010), macropinocytosis (Sánchez et al., 2012) and receptor-mediated endocytosis (Alcamí, Carrascosa, & Viñuela, 1989; Galindo et al., 2015; Hernaez & Alonso, 2010) (Fig. 3). Recently, two studies were developed and may have provided new insights about this topic. While Galindo et al. (2015) defend that the virus enters into macrophages by a classical clathrin- or lipid rafts/caveolae-mediated endocytosis, with special requirements for cholesterol, phosphoinositide-3-kinase (PI3K), actin dynamics and related signalling, Hernaez, Guerra, Salas, & Andrés (2016) showed that ASFV is internalized by both constitutive macropinocytosis and clathrin-mediated endocytosis (Fig. 3).

Concerning the receptor-mediated internalization, although the receptors for the virus remain unknown, the CD163 expression on the surface of swine macrophage has been correlated with susceptibility to ASFV infection (Sánchez-Torres et al., 2003). Additionally, several viral proteins such as p30, p12 and p54 bind to cell surface and are important for viral attachment and internalization (Angulo, Viñuela, & Alcamí, 1993; Gómez-Puertas et al., 1998).

Once inside the cell, ASFV particles move from early endosomes or macropinosomes to late, multivesicular endosomes where the virus becomes uncoated (Valdeira, Bernardes, Cruz, & Geraldes, 1998; Alonso et al., 2013). This gradual maturation of endosomes involves changes in their relative cytoplasmic position closer to the nucleus as well as their luminal environment. The intraluminal acidification of the late endosomes is required for a desencapsidation of the virions, a necessary step for uncoating and for a successful ASFV infection (Cuesta-Geijo et al., 2012). Upon virus uncoating, which involves the disruption of the outer membrane and the protein capsid, the inner membrane becomes exposed and fuses with the endosomal membrane, allowing the viral core egress into the cytosol to initiate replication (Hernaez et al., 2016) (Fig. 3).

ASFV virion reaches the replication site in the perinuclear area, close to the microtubule organization center (MTOC), through microtubules. In fact, the movement of ASFV particles relies on microtubules, and some studies showed a high affinity interaction between ASFV-p54 and the microtubule motor protein dynein, suggesting that this interplay facilitates the virus trafficking (Alonso et al., 2001; Hernaez, Escribano, & Alonso, 2006).

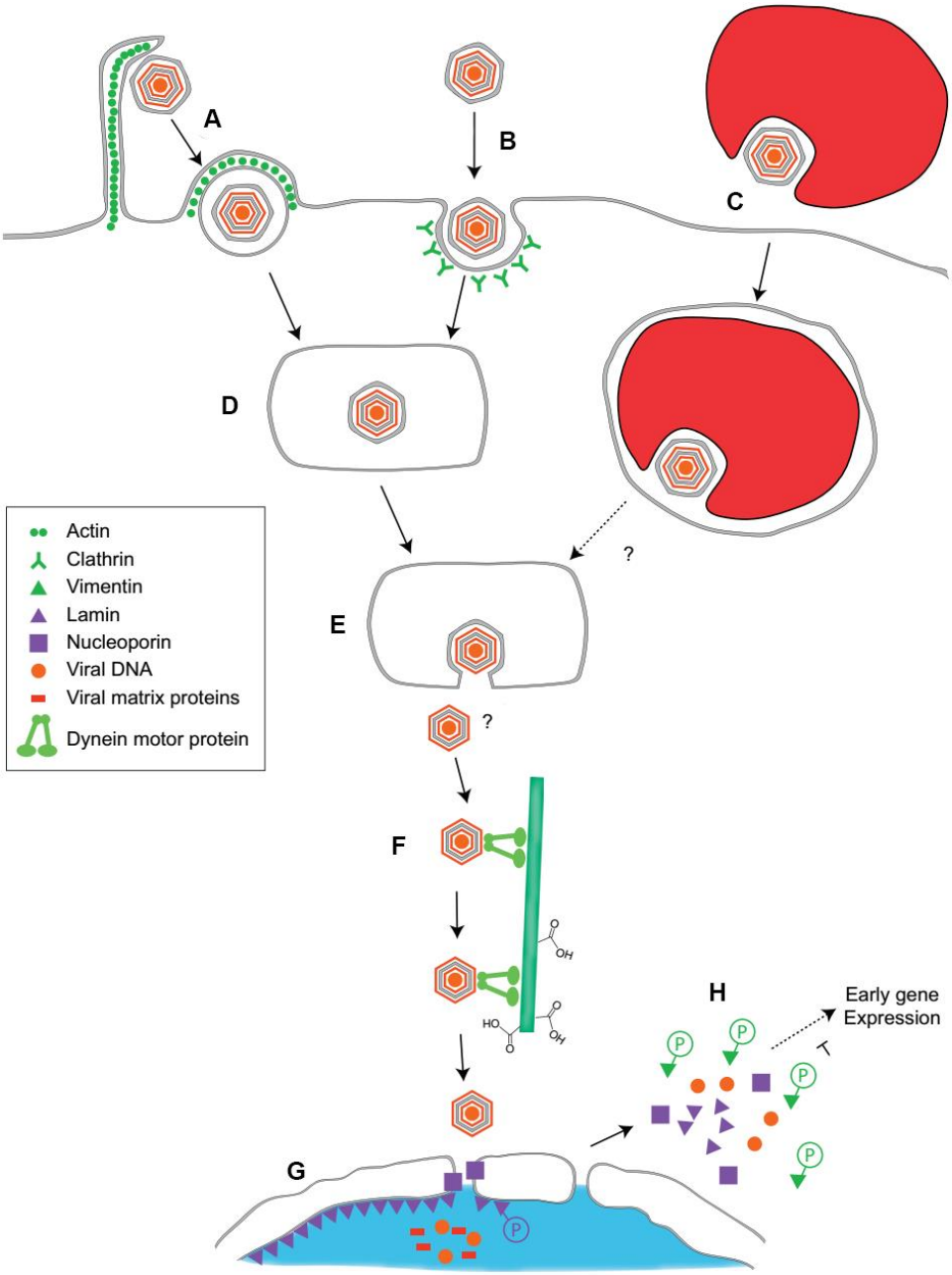

Figure 3. Early events during ASFV infection: ASFV enters cells using macropinocytosis (A) or clathrin coated pits (B). Virus can also enter certain cells attached to phagocytosed red blood cells (C). Post-entry ASFV enters the endosomal–lysosomal system (D) from which it exits by fusing with the membrane and in doing so loses its external envelope (E). Virions are directed to perinuclear regions by microtubules through the interaction between dynein motors and the structural protein p54 (F). Viral matrix proteins and viral DNA enters the nucleus to initiate viral replication and co-incident with this is the phosphorylation and disassembly of nuclear lamins (G). Early viral gene expression and the initial stage of factory formation begins including the recruitment of vimentin and nuclear proteins, and presumably the movement of viral DNA to replication sites (H). Figure and legend were adapted from Netherton and Wileman (2013).

1.5.2. ASFV gene expression and DNA replication

After internalization, viral gene transcription is initiated, using enzymes packaged in the virion core (Salas, Rey-Campos, Almendral, Talavera, & Viñuela, 1986; Dixon et al., 2013). The transcription factors encoded by ASFV recognize a short sequence containing the viral promoter, localized upstream of each gene, that are specific for the different viral stages. ASFV genes expression are indeed strongly temporal regulated, being classified as immediately early, early, intermediate and late genes (Rodríguez & Salas, 2013) (Fig. 4).

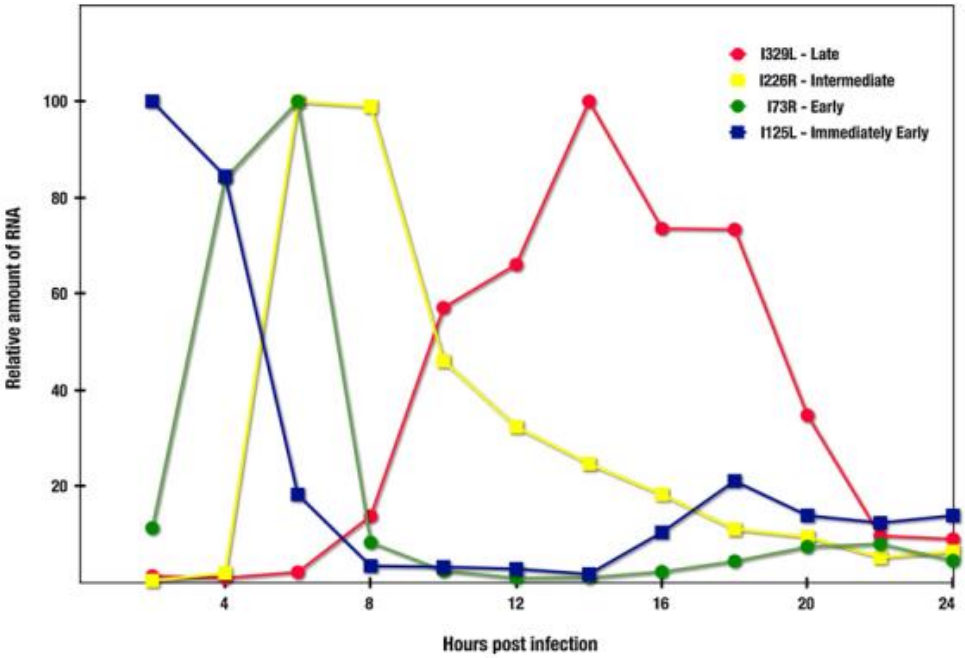

Figure 4. Accumulation kinetics for immediately early (blue, I215L), early (green, I73R), intermediate (yellow, I226R) and late (red, I329L) transcripts throughout ASFV infection. Primer extension assays were used to detect and measure steady state RNA levels for the different transcripts. The quantity of each transcript is plotted as the percentage of the maximum level. Figure and legend were adapted from Rodríguez and Salas (2013).

Immediately early and early genes are expressed prior to DNA replication, however it has been shown that while immediately early genes are repressed before the onset of DNA replication, the expression of early genes is detectable as early as 2 hpi, with a plateau of accumulation at 2-6 hpi, and can be detectable at late times of infection. ASFV early genes encode for enzymes involved in the nucleotide metabolism, DNA replication, regulation of host cell responses to infection, and for transcription factors that are necessary for intermediate and late gene expression (Almazán et al., 1992; Rodriguez, Salas, & Viñuela, 1992; Rodríguez & Salas, 2013).

On the other hand, the expression of intermediate and late ASFV genes are dependent on the viral DNA replication, and their expression is not detected when cells are infected in the presence of inhibitors of DNA replication, like cytosine arabinoside (AraC) (Rodríguez & Salas, 2013). Intermediate transcripts are first detected at 4-6 hpi, coincident with maximum expression of early genes, reaching a maximum level of accumulation at 6-8 hpi, and sharply

decreased at later times. The beginning of late gene expression is coincident with the maximum levels of intermediate mRNAs, reaching a maximum at 12-16 hpi and decreasing slowly thereafter. These two classes of genes codify for structural proteins of the virion, as well as polymerases and early transcription factors, required for the expression of the early genes, that will be packaged into the new virions (Rodríguez, Salas, & Viñuela, 1996; Rodríguez & Salas, 2013; Muñoz-Moreno et al., 2015).

ASFV DNA replication starts with a brief replication phase in the host cell nucleus, following a second replication phase in the cytoplasmic viral factories with a maximum peak at 8 hpi (García-Beato, Salas, Viñuela, & Salas, 1992a; Simões, Martins, & Ferreira, 2015b). Although the intranuclear phase of ASFV infection is still poorly understood, the early intranuclear replication disrupts nuclear structures and modifies the landscape of the host cell nucleus (Ballester et al., 2011; Simões et al., 2015b; Simões, Rino, Pinheiro, Martins, & Ferreira, 2015a). These events provide an environment that favours ASFV replication and may represent a mechanism by which ASFV controls cellular transcription and facilitate viral DNA replication.

The ASFV genome encodes enzymes required for transcription and replication, and virion structural proteins. The known functions of ASFV viral proteins were reviewed in Dixon et al. (2013) and are shown in table 1 and 2.

Gene function	Gene name	Predicted protein size (kDa)
Nucleotide metabolism, transcription, replication and repair		
Thymidylate kinase	A240L	27.8
Thymidine kinase	K196R	22.4
dUTPase*	E165R	18.3
Ribonucleotide reductase (small subunit)	F334L	39.8
Ribonucleotide reductase (large subunit)	F778R	87.5
DNA polymerase family B	G1211R	139.8
DNA topoisomerase type II*	P1192R	135.5
Proliferating cell nuclear antigen (PCNA)-like	E301R	35.3
DNA polymerase X-like*	O174L	20.3
DNA ligase*	NP419L	48.2
AP endonuclease class II*	E296R	33.5
RNA polymerase subunit 2	EP1242L	139.9
RNA polymerase subunit 6	C147L	16.7
RNA polymerase subunit 1	NP1450L	163.7
RNA polymerase subunit 3	H359L	41.3
RNA polymerase subunit 5	D205R	23.7
RNA polymerase subunit 10	CP80R	
TFIIB like	C315R	
Helicase superfamily II	A859L	27.8
Helicase superfamily II	F1055L	123.9
Helicase superfamily II	B962L	109.6
Helicase superfamily II	D1133L	129.3
Helicase superfamily II	Q706L	80.4
Helicase superfamily II	QP509L	58.1
Transcription factor SII	I243L	28.6
Guanylyl transferase*	NP868R	29.9
PolyA polymerase large subunit	C475L	54.8
FTS J-like methyl transferase domain	EP424R	49.3
ERCC4 nuclease domain	EP364R	40.9
Lambda-like exonuclease	D345L	39.4
VV A2L-like transcription factor	B385R	45.3
VV A8L-like transcription factor	G1340L	155.0
VV VLTF2-like late transcription factor, FCS-like finger	B175L	20.3
DNA primase	C962R	111.3
Other enzymes		
Prenyltransferase*	B318L	35.9
Serine protein kinase*	R298L	35.1
Ubiquitin conjugating enzyme*	I215L	24.7
Nudix hydrolase*	D250R	29.9
Host cell interactions		
IAP apoptosis inhibitor*	A224L	26.6
Bcl 2 apoptosis inhibitor*	A179L	21.1
Inhibitor of host gene transcription*	A238L	28.2
C-type lectin-like*	EP153R	18.0
CD2-like. Causes haemadsorption to infected cells*	EP402R	45.3
Similar to HSV ICP34.5 neurovirulence factor	DP71L	8.5
Nif S-like	QP383R	42.5
Phosphoprotein binds to ribonucleoprotein-K	CP204L	30.0

Table 1. Genes encoding ASFV proteins involved in DNA replication, repair, nucleotide metabolism, transcription and other enzymatic activities or host defence evasion. The gene nomenclature is shown in the central column and predicted molecular weight in the Benin 97/1 genome is shown on the right. Asterisks (*) indicate those for which functional data is available. Table and legend were adapted from Dixon et al. (2013).

Gene function	Gene name	Predicted protein size (kDa)
Structural proteins and proteins involved in morphogenesis		
P22	KP177R	20.2
Histone-like	A104R	11.5
P11.5	A137R	21.1
P10	A78R	8.4
pA151R. Component of redox pathway	A151R	17.5
P72 major capsid protein. Involved in virus entry	B646L	73.2
Sulfhydryl oxidase componet of redox pathway	B119L	14.4
P49. Required for formation of vertices in icosahedral capsid	B438L	49.3
Chaperone. Involved in folding of capsid. Not incorporated into virions	B602L	45.3
ERV 1-like. Involved in redox metabolism*	B119L	14.4
SUMO-1-like protease. Involved in polyprotein cleavage	S273R	31.6
pp220 polyprotein precursor of p150, p37, p14 and p34. Required for packaging of nucleoprotein core	CP2475L	281.5
P32 (P30) phosphoprotein. Involved in virus entry	CP204L	23.6
pp62 (pp60) polyprotein precursor of p35 and p15	CP530R	60.5
P12 attachment protein	O61R	6.7
P17. Required for progression of precursor membranes to icosahedral intermediates	D117L	13.1
J5R. Transmembrane domain	H108R	12.5
P54 (j13L). Binds to LC8 chain of dynein, involved in virus entry. Required for recruitment of envelope precursors to the factory	E183L	19.9
J18L. Transmembrane domain	E199L	22.0
P14.5. DNA-binding. Required for movement of virions to plasma membrane	E120R	13.6
E248R (k2R). Possible component of redox pathway required disulphide bond formation Structural protein	E248R	27.5
XP124L. Multigene family 110 member Contains KDEL ER retrieval sequence and transmembrane domain	MGF 110-4L (XP124L)	14.2
EP402R. Similar to host CD2 protein Required for binding red blood cells to infected cells and extracellular virus particles Glycoprotein inserted into external virus envelope	EP402R	45.3

Table 2. Genes encoding structural proteins and other proteins involved in virus morphogenesis. Genes which encode known virus structural proteins and other proteins involved in virus morphogenesis are indicated. The gene name is indicated in the central column and predicted molecular weight in the right column. Table and legend were adapted from Dixon et al. (2013).

1.5.3. ASFV morphogenesis

The assembly of the ASFV particles takes place in viral factories, located in cellular cytoplasmic areas close to the nucleus and MTOC, which resemble aggresomes that are formed in response to misfolded proteins or even as antiviral defence (Wileman, 2007).

These areas are enwrapped in a vimentin cage and surrounded by ER membranes (Andrés, García-Escudero, Simón-Mateo, & Viñuela, 1998) being mitochondria also recruited to their periphery during infection (Rojo et al., 1998).

The steps of ASFV morphogenesis include the formation of the inner envelope, the progressive formation of the capsid on the convex face of the virus inner envelope, the assembly of the core shell on the concave side of the envelope, and the formation of the nucleoid. Concerning the formation of the ASFV nucleoid, although the exact mechanism of this last step in morphogenesis is still unknown, some models suggest that the viral DNA is encapsidated, possibly together with ASFV nucleoproteins p10 and pA104R, and then condensed inside the assembling virus particles (Salas & Andrés, 2013).

1.5.4. Virion egress

Following mature virus particles assembly, intracellular virus are transported from viral factories to the cell surface through cellular microtubules, depending on the conventional kinesin and on the capsid protein ASFV-pE120R (Andrés et al., 2001b; Jouvenet, Monaghan, Way, & Wileman, 2004). Once at the cell surface, ASFV particles are released by budding to yield extracellular enveloped virions (Breese & DeBoer, 1966). On the other hand, cell lysis observed at late times of infection might represent an alternative mechanism of viral egress. ASFV virions can also induce unbranched actin projections that originate from the cellular plasma membrane, which may facilitate cell-cell viral spread (Jouvenet et al., 2006).

2. ASFV histone-like protein

2.1. Overview of the histone-like proteins

ASFV genome codes for a putative histone-like protein (pA104R), that shares a sequence identity of 25 to 30% with two families of bacterial histone-like proteins (HU and IHF), being the only histone-like protein encoded by a eukaryotic virus (Borca et al., 1996; Neilan et al., 1993). Histone-like proteins are small proteins present in bacteria, possessing superficial similarities with eukaryotic histone proteins (basicity, abundance, DNA binding properties and low molecular weight) (Luijsterburg, Noom, Wuite, & Dame, 2006). These proteins, also referred as nucleoid-associate proteins (NAPs), are associated with the bacterial DNA, compacting this structure to fit inside the bacterial cell and increasing the nucleoid stability (Pettijohn & Pfenninger, 1980). These DNA-binding proteins are thought to act as architectural components within the nucleoid and to modulate gene expression.

2.2. Biological function of bacterial histone-like proteins

Contrasting to eukaryotic organisms, where DNA in the nucleus is compacted by histones which organize DNA in a hierarchical process into the higher order structure that is chromatin, the bacterial chromosome is condensed to a form called the nucleoid, but does not possess any higher-order chromosomal organization and represents a comparatively open structure, accessible to DNA-binding proteins, RNA and DNA polymerases throughout the cell cycle (Hobot et al., 1985; Robinow & Kellenberger, 1994). In the case of Escherichia coli, 4.6 Mb of DNA with a contour length of approximately 1.6 mm must be contained and compacted within a cell that measures about 4 μm long and 1 μm wide (Dame, 2005). The packaging of DNA in bacteria is a complex process involving several different mechanisms including cellular confinement, macromolecular crowding, DNA supercoiling by DNA gyrase activity and histone-like protein interactions (Dame, 2005; De Vries, 2010).

Histone-like proteins play an important role in the nucleoid compaction and in the control of its structure. Bacteria mutants lacking functional histone-like proteins have larger decondensed nucleoids (Graumann, 2001; Kano & Imamoto, 1990; Paull, Haykinson, & Johnson, 1993), whereas gain-function mutants result in severe nucleoid compaction and altered bacterial morphology (Fig. 5) (Kar, Edgar, & Adhya, 2005; Macvanin & Adhya, 2012; Spurio et al., 1992). Still the exact mechanism and the contribution of each nucleoid-associate protein to global nucleoid compaction is unclear (Dame & Tark-Dame, 2016).

Furthermore, histone-like proteins are known to perform topological modification of the chromosome (twisting, bending and folding), to regulate the function of promoters of individual operons and control the expression of many genes, affecting bacterial transcription on a global scale (Browning, Grainger, & Busby, 2010). Genes essential for cell viability, involved in bacterial virulence whose expression varies in response to different environmental stimuli, such as changes in temperature, pH, osmolarity, are genes reported to being affected by histone-like proteins (Berger et al., 2010; Blot, Mavathur, Geertz, Travers, & Muskhelishvili, 2006; Kar et al., 2005; Lang et al., 2007; McLeod & Johnson, 2001; Oberto, Nabti, Jooste, Mignot, & Rouviere-Yaniv, 2009).

Although the architectural properties of many histone-like proteins are well characterized *in vitro*, it has been difficult to investigate their roles *in vivo* due to functional, as well as the pleiotropic effect of deletion or overexpression of many of these proteins.

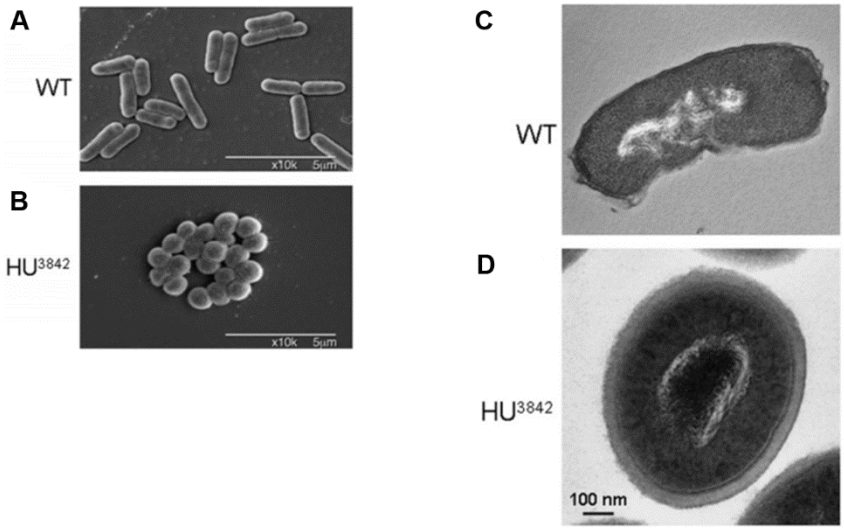

Figure 5. Effects of a gain-function HU mutant on colony morphology and nucleoid compaction. Scanning electron microscopic pictures of wild-type E. coli cells (A) and mutant HU3842 (B). Thin section electron micrographs of wild-type E. coli cells (C) and mutant HU3842 (D). Figures and legend were adapted from Kar et al. (2005) and Macvanin & Adhya (2012).

2.3. Histone-like proteins classification

The major histone-like proteins have different DNA-binding properties and can be divided into two categories, those that have DNA sequence specificity and bind to specific locations of the bacterial chromosome, such as IHF (integration host factor), Lrp (leucine responsive protein), SMC (structural maintenance of chromosomes), CbpA (curved DNA binding protein A), CbpB (curved DNA binding protein B); and those that do not recognise a particular DNA sequence and are located relatively uniformly throughout the nucleoid, as HU (histone-like protein from E. coli strain U93), H-NS (histone-like nucleoid structuring protein), StpA (suppressor of the Td' phenotype), Fis (factor for inversion stimulation) and Dps (DNA binding protein from starved cells) (Azam, Hiraga, & Ishihama, 2000; Prosseda et al., 2002). Interestingly, the intracellular concentration of these proteins varies in a growth phase dependent manner (Dorman, 2013).

The bacterial histone-like proteins can also be categorized according to their structural effect on DNA. Some NAPs (H-NS, SMC, Lrp) are DNA bridging proteins that are likely to play a role in DNA loops formation and stabilization by creating patches of bridged DNA segments along the DNA loop, while others (HU, IHF, Fis) have DNA bending properties and affect the arrangement of the nucleoid in loops (Dame, 2005; Luijsterburg et al., 2006) (Fig. 6). Dps, which is the predominant histone-like protein in cell stationary phase, also forms crystal-like structures with DNA, transforming the nucleoid into a transcriptionally inactive structure that is effectively protected against damage (Frenkiel-Krispin et al., 2004).

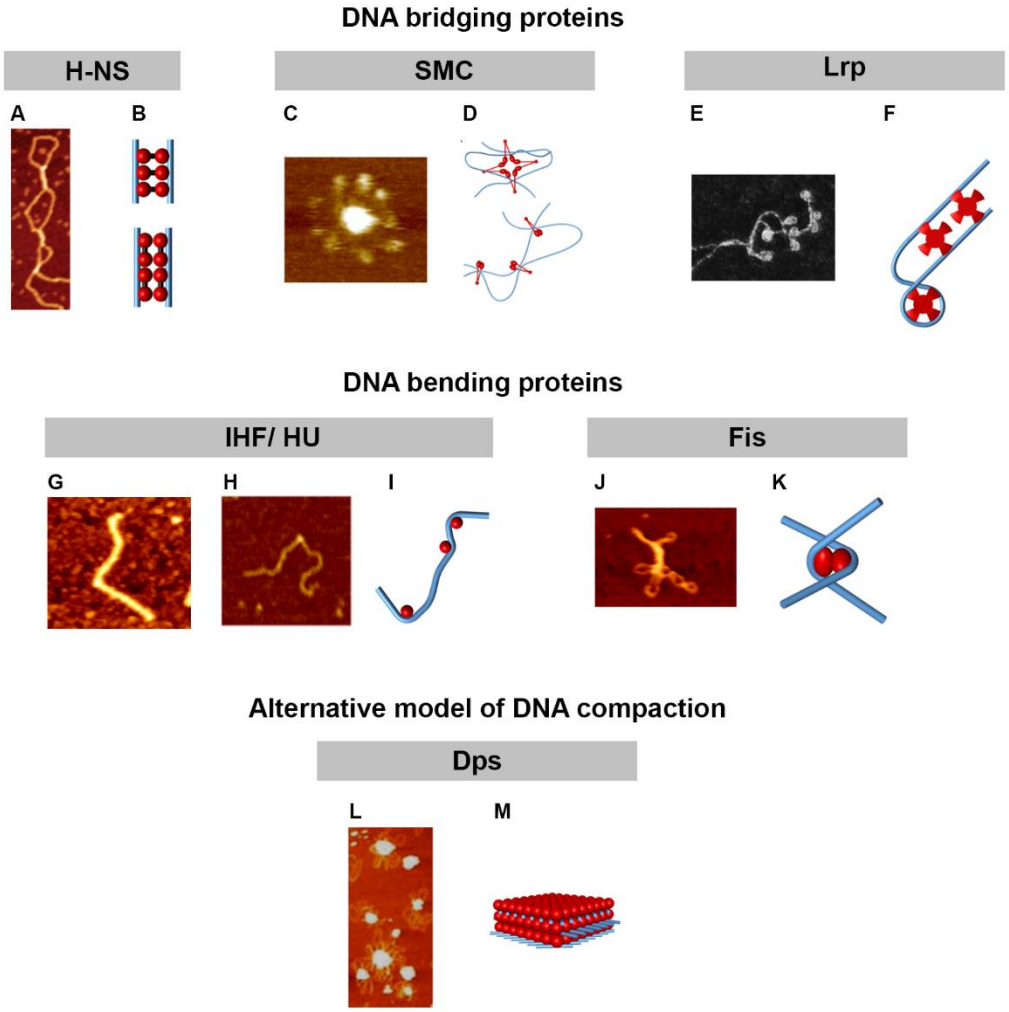

Figure 6. Architectural properties of histone-like proteins.
DNA bridging proteins (A-F): (A) Scanning force microscopy (SFM) image of a DNA loop formed by H-NS as consequence of DNA duplex bridging. (B) Low resolution model for DNA duplex bridging mediated by H-NS. (C) SFM image of Bacillus subtilis SMC that forms a rosette-like structure presumably by joining the head domains (D) Low resolution models for DNA duplex linking due to gathering or trapping by SMC. (E) EM image of the Bacillus subtilis LrpC–DNA complex (L) Low resolution model for DNA duplex bridging and wrapping mediated by Lrp. DNA bending proteins: (G) SFM image of an IHF–DNA complex (H) SFM image of HU–DNA complexes, when the presence of low concentrations of HU. (I) Low resolution model for DNA compaction by the binding of multiple HU/IHF molecules. (J) SFM image of Fis–DNA complexes with Fis bound at the nodes of supercoiled pUC18 plasmids. (k) Low resolution model for node formation due to Fis–Fis interactions. Alternative mechanisms of organization and compaction: (L) SFM image of Dps–DNA complexes. (M) Low resolution model of three-dimensional hexagonal Dps–DNA arrays. Figures and legend were adapted from Beloin et al. (2003); Ceci et al. (2004); Dame et al. (2005); Frenkiel-Krispin et al. (2004); Luijsterburg et al. (2006); Mascarenhas et al. (2005); Schneider et al. (2001); van Noort, Verbrugge, Goosen, Dekker, & Dame (2004).

3. Viral infections and cellular epigenetic remodelling

Viruses have co-evolved and share a unique relationship with their hosts, developing numerous mechanisms to evade host's natural defences. Studies on the role of host cellular chromatin in the regulation of viral infection have uncovered the importance of viral-host chromatin interactions in the establishment of viral infection (Adhya & Basu, 2010; Knipe et al., 2013). Moreover, viral proteins by interacting with chromatin remodelling factors elicit various epigenetic changes in host cells in order to control gene expression and host innate immune antiviral defence processes, thereby promoting robust virus replication and pathogenesis. HIV, Epstein-Barr virus (EBV), hepatitis B virus (HBV), human papillomavirus, simian virus 40 (SV40), bovine leukemia virus (BLV), herpes simplex virus (HSV) and dengue virus are some currently known viruses that exhibit epigenetic immune evasion mechanism to survive and propagate in their host (Adhya & Basu, 2010).

3.1. Chromatin organization and structure

3.1.1. Histones and nucleosome structure

In eukaryotic cells, genomic DNA is packaged by histones into chromatin to fit inside the nucleus. The fundamental unit of chromatin is the nucleosome, consisting of 146 base pairs (bp) of DNA wrapped around a histone octamer containing two copies each of the histones H2A, H2B, H3 and H4 (Luger, Mäder, Richmond, Sargent, & Richmond, 1997). Linker histone (H1/H5) binding organizes an additional 20 bp of DNA to complete the nucleosome containing ~167 bp of DNA and it is responsible for forming the chromatosome structure (Thoma, Koller, & Klug, 1979; Woodcock, Skoultchi, & Fan, 2006). Each nucleosome is linked to the next by small segments of linker DNA, representing a primary packing structure. Further condensation is achieved through the formation of the more compact and repressive 30-nm chromatin fibre (secondary structure) and several more levels of higher-order chromatin organization (Jansen & Verstrepen, 2011; Luger, Dechassa, & Tremethick, 2012; Szerlong & Hansen, 2011) (Fig. 7).

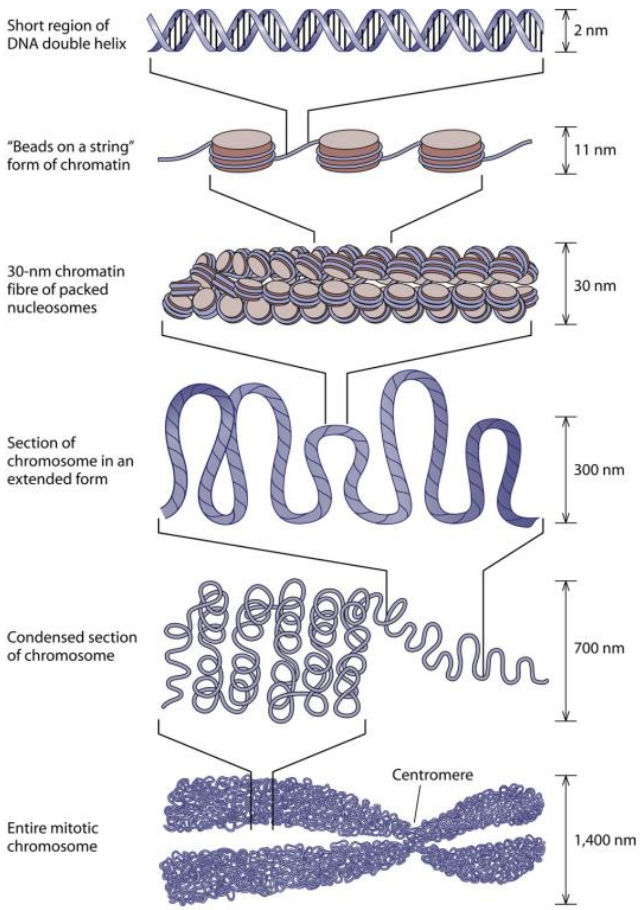

Figure 7. The organization of DNA within the chromatin structure.
DNA is wrapped around a histone octamer to form nucleosomes. Nucleosomes are connected by stretches of linker DNA. This basic nucleosome structure is folded into a fibre about 30 nm in diameter and these fibres are then further folded into higher-order structures. Figures and legend were adapted from Felsenfeld & Groudine (2003) and Jansen & Verstrepen (2011).

During DNA replication and gene transcription, the DNA-binding transcription factors and RNA polymerase II must access DNA, however the DNA compaction into chromatin restricts this contact. Thus, it has been clear that the plasticity and modulation of chromatin structure play a crucial role in the regulation of transcription in eukaryotes and other biological processes inherent to DNA (Hübner, Eckersley-Maslin, & Spector, 2013; Li & Reinberg, 2011).

3.1.2. Euchromatin and heterochromatin

From the functional point of view, various chromatin structures are commonly divided into euchromatin and heterochromatin. Euchromatin is less condensed, more accessible and corresponds generally to genome regions that possess actively transcribed genes, whereas heterochromatin is typically highly condensed, inaccessible and correlated to transcriptionally inactive regions of genome (Grewal & Jia, 2007; Quina, Buschbeck, & Di Croce, 2006). The heterochromatin domains, which can be divided into constitutive and facultative heterochromatin depending respectively if these regions remain condensed throughout the cell cycle or can change in response to cellular signals and gene activity, are characterized by the

enrichment of histone H3 methylated at lysine 9 (H3K9me) and heterochromatin protein HP1 (Eskeland, Eberharter, & Imhof, 2007; Grewal & Jia, 2007; Peng & Karpen, 2009; Quina et al., 2006; Stewart, Li, & Wong, 2005). By its turn, the acquisition of active chromatin marks, such as H3K9 acetylation and H3K4me2, leads to euchromatin formation (Bártová, Krejčí, Harničarová, Galiová, & Kozubek, 2008; Quina et al., 2006).

, These chromatin domains are relatively well organized in the eukaryotic cells nucleus, the heterochromatin tends to be localized with the nucleolus and nuclear periphery, while euchromatin tends to reside at the interior of the nucleus (Bártová et al., 2008; Margueron & Reinberg, 2010).

3.2. Chromatin remodelling

Nucleosomes on their own are not inert structures but dynamically fluctuate, affecting the overall chromatin structure and thereby regulating DNA accessibility (Bowman & Poirier, 2015). The regulation of chromatin structure is indeed a complex and dynamic process, regulated by epigenetics changes that are governed by various types of determinants, including DNA methylation patterns, nucleosome remodelling and noncoding RNAs (ncRNAs), and histone modifications (Cao et al., 2015).

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3.2.1. Histone modifications

The histone modification, either by posttranslational modifications (PTMs) or by the introduction of histone variants, play fundamental roles on gene transcription and in other DNA processes (e.g. repair, replication and recombination) (Bannister & Kouzarides, 2011; Campos & Reinberg, 2009). Histones are subjected to different PTMs that can be dynamically added and removed enzymatically, with the best-studied modifications including acetylation, methylation, phosphorylation, ubiquitylation, and ADP-ribosylation (Table 3) (Bártová et al., 2008; Patel & Wang, 2013; Rothbart & Strahl, 2014). These modifications are located on both the tails and core of the histone octamer, and affect the unwrapping dynamics or the core stability of the nucleosome (Bowman & Poirier, 2015; Kouzarides, 2007; Karolin Luger, 2006). PTMs not only regulate chromatin structure, but they also recruit other chromatin remodelling factors (Bannister & Kouzarides, 2011; Bowman & Poirier, 2015).

Modification	Role in transcription	Modification site
Acetylation	Activation	H3ac, H3K9ac, H3K14ac, H3K27ac
Methylation	Activation	H3K4me1, H3K4me2, H3K4me3, H3K36me3, H3K79me2
Methylation	Repression	H3K9me3, H3K27me3
Phosphorylation	Activation	H3S10

Table 3. Some histone modification and their role in transcription.
Table and legend was adapted from Schafer & Baric (2017).

3.2.2. Histone deacetylases (HDACs) and histone acetyltransferases (HATs)

Among the histone PTMs, acetylation of core histones is probably the best understood type of modification and the most associated with promoting transcription (de Ruijter, van Gennip, Caron, Kemp, & van Kuilenburg, 2003). Histone acetylation occurs at the Ɛ-amino groups of lysine residues located in amino-terminal domains of the histones, also referred to as histone tails because they protrude out from the nucleosome (de Ruijter et al., 2003; Roth, Denu, & Allis, 2001). Important positions are K9 and K14 of histone H3, and K5, K8, K12, and K16 of histone H4 (Bjerling et al., 2002). The acetylation of histones neutralizes their positive charges and loses their interaction with negatively-charged DNA. This opens the chromatin structure to facilitate the binding of transcription factors and, subsequently, gene transcription (Bolden, Peart, & Johnstone, 2006).

The histone acetylation pattern, which is key epigenetic regulator of chromatin structure and gene expression, is determined by two group of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). The acetylation of lysine residues by HATs enzymatic activities induces an open chromatin state, granting the access of regulatory proteins to DNA, and thereby is generally linked to an active transcription, while the reverse modification, catalysed by HDACs results in transcriptional repression (Fig. 8) (Herbein & Wendling, 2010; Yang & Seto, 2007). The non-histone proteins [e.g. p53, ataxia-telangiectasia mutant (ATM), heat shock protein 90, α-tubulin] are also target of HATs and HDACs and their modifications seem to play an important role on a cellular level (Table 4) (Glozak, Sengupta, Zhang, & Seto, 2005).

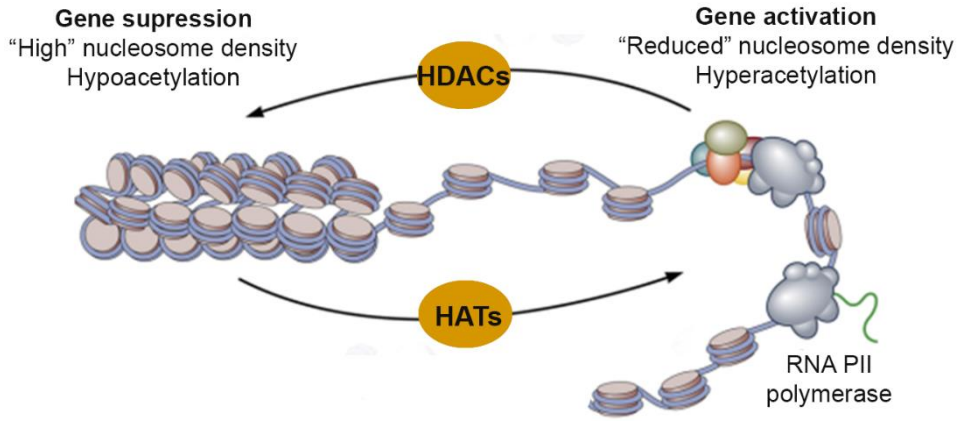

Figure 8. The dynamic state of histone acetylation/ deacetylation regulated by HDACs and HATs. Acetylation of histones alters accessibility of chromatin and allows gene transcription. Figure was adapted from Kristie (2016).

Pathway	Protein
Cell motility	α-Tubulin, cortactin
Chaperones	HSP90, HSP70
Gene transcription factors and co-regulators	p53, p73, GATA-1, 2 and 3, MyoD, E2F1, 2 and 3, PLAG-1 and PLAGL-2, c-myc, BCL-2, Rb, PCC-1α
Chromatin structure	HMG-A1, B1, B2, N1 and N2, SRY
Nuclear receptors (DNA)	Androgen receptor, glucocorticoid receptor, estrogen receptor α
Signalling mediators	SAT3, Smad7, β-Catenin
DNA repair	Ku70, WRN
Nuclear import	Importin-α7
Inflammation mediator	HMGB1
Viral protein	E1A, L-HDAg, S-HDAg

Table 4. Non-histone substrates of HDACs (short list).
Table and legend were adapted from Marks & Xu (2009).

To date, the HDAC family contains 18 known isoforms organized into 4 classes of enzymes: Class I (HDAC 1, 2, 3 and 8); Class II (subdivided in subgroup IIa - HDAC 4, 5, 7 and 9, and IIb – HDAC 6 and 10); Class III (sirtuins), and Class IV (HDAC 11) (Bolden et al., 2006; de Ruijter et al., 2003). HATs are classified into several families based on their structural homologies: Gcn5-related N-acetltransferases, GNAT [e.g. Gcn5 (general control non-derepressible 5), HAT1 and PCAF (p300/CBP-associated factor)]; p300/CBP (adenoviral E1A-associated protein of 300 kDa and CREB-binding protein); and MYST proteins (e.g. TIP60, MOZ, MORF, HBO1 and MOF) (Avvakumov & Côté, 2007; Dyda, Klein, & Hickman, 2000; Roth et al., 2001; Yang & Seto, 2007).

3.2.2.1. Histone deacetylase inhibitors (HDACi) and histone acetyltransferase inhibitors (HATi)

A large number of histone deacetylase inhibitors (HDACi) and histone acetyltransferases inhibitors (HATi) have been obtained from natural sources or synthetically developed. Concerning HDACs, class I, II and IV can be pharmacologically modulated by HDACi. These inhibitors can be structurally grouped into several classes, including hydroxamates, cyclic peptides, short-chain fatty acids, benzamides and electrophilic ketone (Bolden et al., 2006; Kim & Bae, 2011) (Table 5). Since altered expression of HDACs isoforms has been reported in association with cancer disease (Barneda-Zahonero & Parra, 2012; Falkenberg & Johnstone, 2014; Marks & Xu, 2009; Ropero & Esteller, 2007), a lot of efforts are currently being devoted to the development of safe, efficient and selective HDACi. More than 40 HDACi were indeed obtained and are currently under development and study (Zwergel, Stazi, Valente, & Mai, 2016), being vorinostat, romidepsin, belinostat and panobinostat approved by FDA for the treatment of relapsed cutaneous T-cell lymphoma (CTCL), CTCL and peripheral T-cell lymphoma (PTCL), relapsed or refractory PTCL and myeloma, respectively (Lee et al., 2015; Mann, Johnson, Cohen, Justice, & Pazdur, 2007; Raedler, 2016; VanderMolen, McCulloch, Pearce, & Oberlies, 2011).

Although the HDACi are mostly studied as anticancer agents, there is a growing number of studies demonstrating that HDACs play a crucial role in other diseases such as neurological disorders (Gray, 2011), inflammatory processes (Dinarello, 2010), parasitosis (Bougdour et al., 2009; Chaal, Gupta, Wastuwidyaningtyas, Luah, & Bozdech, 2010) and viral infections (Archin et al., 2012; Ghosh, Perrine, Williams, & Faller, 2012; Huber et al., 2011; Michaelis et al., 2005; Radkov et al., 1999).

Class	Compound	[range]	HDAC specificity
Short-chain fatty acid	Butyrate	mM	Class I, IIa
	Valproic acid (VPA)	mM	Class I, IIa
	AN-9 (prodrug)	µM	N/A
Hydroxamate	Trichostatin A (TSA)	nM	Class I, II
	Suberoylanilide hydroxamic acid (SAHA, Vorinostat)	µM	Class I, II
	PXD101	µM	Class I, II
	Oxamflatin	µM	Class I, II
	LAQ824	nM	Class I, II
	LBH589	nM	
	m-carboxycinnamic acid bishydroxamide (CBHA)	µM	Class II
	Scriptaid	µM	Class I, II
	Pyroxamide	µM	Class I, unknown effect on class II
	Suberic bishydroxamic acid (SBHA)	µM	HDAC 1 and 3
	Azelaic bishydroxamic acid (ABHA)	µM	N/A
	SK-7041	nM	HDACs 1 and 2
	SK-7068	nM	HDACs 1 and 2
	CG-1521	µM	N/A
	Tubacin	µM	Class IIb
Benzamide	MS-275	µM	HDACs 1, 2, 3, 8
	CI-994 (tacedinaline)	µM	Class I
Cyclic tetrapeptide	Depsipeptide	nM	Class I
	Trapoxin A	nM	Class I, IIa
	Apicidin	nM	HDACs 1 and 3, not HDAC8
	CHAPs	nM	Class I
Electrophilic ketone	Trifluoromethylketone	µM	N/A
Miscellaneous	Depudecin	µM	Class I, unknown effect on class II
	MGCD-0103		Class I

N/A, not available

Table 5. Molecular characteristic and HDAC specificity of some HDACi.
Table and legend were adapted from Bolden et al. (2006).

On the other hand, there is little information available on inhibitors of HAT. Several compounds with HAT-inhibitory activity were identified. Curcumin, anacardic acid, garcinol and (-)-epigallocatechin gallate (EGCG) are some naturally occurring HATi (Balasubramanyam et al., 2004b; Balasubramanyam et al., 2004b; Balasubramanyam, Swaminathan, Ranganathan, & Kundu, 2003; Choi et al., 2009). Recently, other new molecules that selective inhibits HATs have been developed, such as NU9056 (Tip60 inhibitor), L002 (p300 inhibitor), C646 (p300/CBP inhibitor) CPTH2 (Gcn5 inhibitor) (Bowers et al., 2010; Chimenti et al., 2009; Coffey et al., 2012; Yang et al., 2013). Interestingly, studies showed that some HATi are potential

anticancer therapeutics (Coffey et al., 2012; Di Martile et al., 2016; Eliseeva, Valkov, Jung, & Jung, 2007; Gao et al., 2013; Yang et al., 2013) and even demonstrated antiviral activities against hepatitis C virus, HIV, chikungunya and vesicular stomatitis virus infections (Hundt, Li, & Liu, 2015; Mantelingu et al., 2007; Von Rhein et al., 2016).

3.2.2.2. HDACs and HATs enzymes and viral infections

Viruses modulate host cell chromatin machinery in order to regulate the expression of their genes in a program that supports a productive infection or allows a viral latent-persistent state (Kristie, 2016). HDACs and HATs are involved in the regulation of the replication of numerous viruses (Herbein & Wendling, 2010; Lieberman, 2006) and interestingly some studies identified viral proteins that interact with, activate or inhibit these chromatin remodelling enzymes (Table 6) (Chiocca et al., 2002; Horwitz et al., 2008; Lang & Hearing, 2003; Punga & Akusjärvi, 2000; Radkov et al., 1999; Valls et al., 2007; Wurm, Wright, Polakowski, Mesnard, & Lemasson, 2012). HDACi treatment indeed lead to the reactivation of latent viruses such as HIV, Epstein-Barr Virus and human cytomegalovirus (Archin et al., 2012; Ghosh et al., 2012; Huber et al., 2011; Michaelis et al., 2005; Radkov et al., 1999) and reduce replication of some enveloped viruses (Vázquez-Calvo et al., 2013; Vázquez-Calvo, Saiz, Sobrino, & Martín-Acebes, 2011). Concerning ASFV, Granja, Sabina, Salas, Fresno, & Revilla (2006) and Simões et al., (2015a) showed that this virus interacts to p300 transactivation and to cellular HDACs, respectively.

All the above-mentioned studies emphasize the importance of host chromatin structure modulation in viral infections and make these inhibitors attractive templates to design new antiviral compounds.

Virus	Gene/ Protein	Cellular targets	Function or structure
SV40	T antigen	p300, CBP	HAT, bromo
Adenovirus	E1A	p300, CBP	HAT, bromo
	E1A	P/CAF	HAT
	GAM1	HDACs 1, 2 and 3	Inhibition of HDACs
HPV	E7	Mi2 (NURD)	Chromatin remodelling
	E7	p300, CBP	HAT
	E2	BRD4	Bromo
HSV	VP16	HCF	H3 Lys4 methylation
	VP22	SETa, TAF-Iα	Histone chaperone
	ICP0	Daxx	Heterochromatin formation?
EBV	Zta (ZEBRA/EB1)	p300, CBP	HAT
	EBNA2	INI1/SNF5	Chromatin remodelling
KSHV	Rta	PARP1	Linker histone, PARP
	LANA	Histone H1	Linker histone
	LANA	BRD2	Bromo
CMV	IE1	HDACs	Inhibition of HDACs
	IE1	Daxx	Heterochromatin
HIV	INT	INI1/SNF5	Chromatin remodelling
	INT	p300	HAT
	TAT	pTEFb	Histone methylation
	TAT	p300, CBP, GCN5, P/CAF, TIP60	HAT
HTLV-I	TAX	p300, CBP	HAT
Influenza	vRNPs	Histone tails	Nuclear matrix
	M1	Histones	Interactions?

Table 6. Examples of viral proteins and their chromatin-associated targets.
Table and legend were adapted from Lieberman (2006).

4. Objectives

In recent years, research developed at the Infectious Disease Laboratory at FMV, ULisboa has been focused on the identification and characterization of ASFV proteins involved in viral transcription and replication, aiming at improving knowledge on ASFV biology and laying the foundations for the development of a protective vaccine(s) and potential use of drugs against the disease.

African swine fever virus ORF A104R has been previously described as having homology to bacterial histone-like protein (Borca et al., 1996; Neilan et al., 1993). However, and despite of this finding, no further research has been pursued, at our knowledge, regarding its biochemical characterization and biological relevance in ASFV infection.

Taking this into consideration, this work aims at evaluating the function of pA104R and its role on ASFV replication, towards its use as a defective infectious single cell cycle (DISC) particle vaccine, and furthermore investigating epigenetic mechanisms involved in ASFV infection and to screening different pharmacologic compounds that could be useful to control ASFV infection.

Therefore, the core objectives of this work are:

- To study the biologic activity of recombinant pA104R, through cloning, expression, purification and characterization of its *in vitro* activity;
- To understand the functional relevance of two conserved residues of pA104R;
- To assess mRNA and protein levels, as well as the intracellular localization of pA104R in ASFV-infected cells, at different time-points of infection;
- To develop a strategy that allows the deletion of the ORF A104R from ASFV genome, in order to obtain defective infectious single-cycle (DISC) vaccine.
- To better understand how ASFV modulates the host epigenetic mechanisms through the evaluation of histone acetylation levels on ASFV-infected cells;
- To screen the effect of histone deacetylase inhibitors on ASFV infection.

The research outcomes of this work represent Chapters II-IV, while major conclusions and future directions of the work will be entailed in Chapter V.

CHAPTER II

DNA-binding properties of the African swine fever virus pA104R, a histone-like protein involved in viral replication and transcription

Gonçalo Frouco, Ferdinando B. Freitas, João Coelho, Alexandre Leitão, Carlos Martins and Fernando Ferreira. (2017). *Journal of Virology,* 91(12), pii:e02498-16.

Abstract

African swine fever virus (ASFV) codes for a putative histone-like protein (pA104R) with extensive sequence homology to bacterial proteins that are implicated in genome replication and packaging. Functional characterization of purified recombinant pA104R revealed that it binds to single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) over a wide range of temperatures, pH values, and salt concentrations and in an ATP-independent manner, with an estimated binding site size of about 14 to 16 nucleotides. Using site-directed mutagenesis, the arginine located in pA104R's DNA-binding domain, at position 69, was found to be relevant for efficient DNA-binding activity. Together, pA104R and ASFV topoisomerase II (pP1192R) display DNA-supercoiling activity, although none of the proteins by themselves do, indicating that the two cooperate in this process. In ASFV-infected cells, A104R transcripts were detected from 2 h postinfection (hpi) onward, reaching a maximum concentration around 16 hpi. pA104R was detected from 12 hpi onward, localizing with viral DNA replication sites and being found exclusively in the Triton-insoluble fraction. Small interfering RNA (siRNA) knockdown experiments revealed that pA104R plays a critical role in viral DNA replication and gene expression, with transfected cells showing lower viral progeny numbers (up to a reduction of 82.0%), lower copy numbers of viral genomes (78.3%), and reduced transcription of a late viral gene (47.6%). Taken together, our results strongly suggest that pA104R participates in the modulation of viral DNA topology, probably being involved in viral DNA replication, transcription, and packaging, emphasizing that ASFV mutants lacking the A104R gene could be used as a strategy to develop a vaccine against ASFV.

Importance

Recently reintroduced in Europe, African swine fever virus (ASFV) causes a fatal disease in domestic pigs, causing high economic losses in affected countries, as no vaccine or treatment is currently available. Remarkably, ASFV is the only known mammalian virus that putatively codes for a histone-like protein (pA104R) that shares extensive sequence homology with bacterial histone-like proteins. In this study, we characterized the DNA-binding properties of pA104R, analyzed the functional importance of two conserved residues, and showed that pA104R and ASFV topoisomerase II cooperate and display DNA-supercoiling activity. Moreover, pA104R is expressed during the late phase of infection and accumulates in viral DNA replication sites, and its downregulation revealed that pA104R is required for viral DNA replication and transcription. These results suggest that pA104R participates in the modulation of viral DNA topology and genome packaging, indicating that A104R deletion mutants may be a good strategy for vaccine development against ASFV.

Keywords: African swine fever virus; DNA binding; histone-like protein; pA104R; viral replication; viral transcription.

2.1. Introduction

African swine fever virus (ASFV) causes a highly lethal disease that is considered one of the most threatening diseases for pig husbandry, with no vaccine or antiviral treatment available yet. Originally endemic in Sub-Saharan Africa, African swine fever disease was introduced in the Transcaucasian countries (Georgia, Armenia and Azerbaijan) and in the Russian Federation (2007), where it is maintained (Sánchez-Vizcaíno et al., 2013). In the course of the last few years, new outbreaks of ASFV have occurred in Ukraine (2012, 2014 and 2015); Belarus (2013); and Lithuania, Estonia, Latvia and Poland (2014 and 2015) (Gallardo et al., 2015).

ASFV infects domestic and wild suids, as well as soft ticks of the genus Ornithodoros, and can be transmitted either directly by contact with infected animals or indirectly via tick bites and through virus-contaminated feed or fomites (Costard et al., 2013). While warthogs and bushpigs are asymptomatic carriers of the disease, acting as reservoirs of infection, the clinical signs in domestic pigs and wild boars may vary from acute to chronic forms, depending on the virulence of the ASFV strain and on the immunological status of the host (Blome et al., 2013; Costard et al., 2013).

ASFV is the only member of family *Asfarviridae* sharing some functional and structural similarities with other large eukaryotic viruses (e.g. poxviruses and iridoviruses), all belonging to the nucleocytoplasmic Large DNA Viruses (NCLDV) clade (King, Adams, Carsten, & Lefkowitz, 2012). Its double-stranded DNA genome ranges from 170 to 190 kbp, and includes between 151 and 167 open reading frames (ORFs) (Dixon et al., 2013), including ORF A104R. This gene codes for a putative histone-like protein (pA104R), highly conserved among all ASFV isolates, that shares a sequence identity of 25 to 30% with two families of bacterial histone-like proteins (HU and IHF), and is the only histone-like protein encoded by a eukaryotic virus (Borca et al., 1996; Neilan et al., 1993). In addition, pA104R possesses the signature of histone-like proteins at residues 57 to 76 of its sequence (PROSITE PS00045), including 8 amino acid residues responsible for DNA interaction and conserved in bacterial histone-like proteins. In prokaryotes, these nucleoid-associated proteins are involved in DNA supercoiling and also play an important role in DNA replication, repair, recombination and transcription (Hashimoto, Imhoff, Ali, & Kow, 2003; Kar et al., 2005; Koli, Sudan, Fitzgerald, Adhya, & Kar, 2011; Oberto et al., 2009) by interacting with topoisomerases (Bensaid, Almeida, Drlica, & Rouviere-Yaniv, 1996; Ghosh, Mallick, & Nagaraja, 2014; Malik, Bensaid, Rouviere-Yaniv, & Drlica, 1996; Wang, 2002). Besides their structural and sequence similarities to eukaryotic histones, these bacterial proteins are often termed histones due to their DNA-binding properties, low molecular weights, and high bending capacities (Grove, 2011; Luijsterburg et al., 2006; Rouvière-Yaniv & Gros, 1975).

In ASFV, it was demonstrated that pA104R binds to double-stranded DNA (dsDNA)- and single-stranded DNA (ssDNA)-cellulose columns (Borca et al., 1996), and although it was

anticipated that pA104R might be involved in DNA-related processes or, alternatively, act as a transcription factor modulating viral gene expression (Borca et al., 1996; Neilan et al., 1993), its activity and biological role remain to be characterized. Therefore, in this study, we aimed to assess pA104R DNA-binding activity using electrophoretic mobility shift assay (EMSA); to understand the functional importance of two conserved residues, both located within the DNA-binding motif, by using site-directed mutagenesis; to verify whether pA104R has DNA-supercoiling activity; and to measure the mRNA and the protein expression levels of A104R, as well as the pA104R distribution pattern, using ASFV-infected Vero cells. Finally, RNA interference (RNAi) assays were performed to clarify the role of pA104R in infection.

2.2. Material and Methods

2.2.1. Cloning, expression, and purification of recombinant A104R^{wt}, A104R^{R69A}, A104R^{R69K} and A104R^{P74A}

The complete ORF A104R, without the stop codon, was PCR amplified from Ba71V genomic DNA, using the A104R_Fw and A104R_Rev primers (Table 7), which contain NdeI and XhoI restriction endonuclease sites at their 5' ends. The resulting PCR product was purified using the High Pure Viral Nucleic Acid kit (Roche) and then cloned in the vector pET24a (Novagen). The three single point mutants (R69A, P74A, and R69K) were generated using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies), following the manufacturer's instructions.

For expression of the recombinant proteins, the Escherichia coli strain BL21(DE3)-pLysS (Novagen) was transformed with either a pET24a/A104R_6XHis wild-type or single-point-mutated A104R version and grown in LB medium (10 g tryptone, 5 g Select yeast extract, 5 g NaCl, pH 7.2) supplemented with kanamycin (30 µg/ml) plus chloramphenicol (34 µg/ml) at 37°C with shaking at 200 rpm until the optical density at 600 nm (OD600) reached 0.1 to 0.2, measured with a Nanodrop 2000 (Thermo Scientific). Induction of protein expression was carried out by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Five hours after induction, bacterial cells were harvested by centrifugation (10,000 X g for 10 min; 4°C) and washed with sterile water. The pellet was resuspended in binding buffer (20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole, pH 7.4). and the cells were lysed by addition of a lysis solution (0.2 mg/ml lysozyme, 20 µg/ml DNase, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and sonicated 5 times for 5 min each time on ice (5 cycles; 70% amplitude). Then, the lysates were centrifuged at 3,000 X g for 15 min, and the pellets were discarded. The extracts were then filtered through a 0.45- µm syringe filter (Rotilabo; Carl Roth) and incubated with Ni Sepharose 6 Fast Flow slurry (GE Healthcare) for 1 h, according to the manufacturer's instructions. The mixture was loaded onto a PD-10 column (GE Healthcare) and washed with binding buffer solution (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) containing increasing concentrations of imidazole (40, 60, and 80 mM), and the

recombinant protein was eluted with elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). The fractions were collected in low-binding tubes and analyzed by SDS-PAGE, and the recombinant pA104R, purified under native conditions, was stored at -80°C until further use.

2.2.2. Electrophoretic mobility shift assay (EMSA)

Biotin-labeled ssDNA molecules with different lengths (10, 20, 30, 40, and 50 nt) were synthetized by Stab Vida, Lda (Lisbon, Portugal), and the double-stranded oligonucleotides were obtained by hybridization with complementary unlabeled molecules as described by Loregian, Sinigalia, Mercorelli, Palù, and Coen (2007). Briefly, equal amounts of complementary strands were mixed in annealing buffer (10 mM Tris, pH 7.8, 50 mM NaCl, and 1 mM EDTA), denatured at 95°C for 5 min, and annealed by gradual cooling to RT. The oligonucleotides (1 pmol) were then incubated with either purified wild-type pA104R or pA104R point mutants (pA104R^{R69A}, pA104R^{R69K}, or pA104R^{P74A}), at different concentrations, in an EMSA buffer (20 µl) containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM EDTA, 5% (vol/vol) glycerol for 30 min at RT. After the addition of 5 X loading buffer, the reaction products were subjected to SDS-PAGE electrophoresis (8 to 16%) (Bio-Rad) in a 0.5X Tris-borate-EDTA (TBE) buffer (Sigma-Aldrich) and electrophoretically transferred onto a positively charged nylon membrane (Amersham Hybond-N$^+$; GE Healthcare). The DNA was cross-linked to the membrane with a UVC 500 linker UV chamber (700 mJ/cm^2; 15 min; Hoefer). Then, the membranes were blocked overnight at 4°C with phosphate-buffered saline (PBS) plus 0.1% (vol/vol) Tween 20 (PBST) (Sigma-Aldrich) containing 5% (wt/vol) bovine serum albumin (BSA) (Sigma-Aldrich), followed by incubation with horseradish peroxidase (HRP)-conjugated streptavidin antibody (RPN1231; GE Healthcare) for 1 h at RT. After five wash steps with PBST (10 min each), chemiluminescence detection was performed with a Clarity Western ECL Substrate detection kit (Bio-Rad) according to the manufacturer's instructions, using Amersham hyperfilms (GE Healthcare).

2.2.3. Supercoiling assay

The DNA-supercoiling activity of pA104R was assayed by monitoring the conversion of relaxed pBR322 DNA (TopoGen) to its supercoiled form, both in the presence and absence of ASFV DNA type II topoisomerase (pP1192R), purified as described by Coelho, Ferreira, Martins, & Leitão (2016). In order to perform the assay, relaxed pBR322 plasmid (150 ng) was incubated with increasing amounts of pA104R and, where indicated, in the presence of pP1192R (0.01 µM) at 37°C, using a reaction buffer (50 mM Tris, pH 7.5, 75 mM NaCl, 6 mM MgCl 2, 1 mM dithiothreitol [DTT], 2 mM ATP) previously optimized for pP1192R activity assays (Coelho et al., 2016) and confirmed to be suitable for pA104R-binding activity, in a final volume of 20 µl. Reactions were stopped by the addition of 5 µl of a stop solution containing 5% (vol/vol) SDS,

0.003% (wt/vol) bromophenol blue, and 25% (vol/vol) glycerol. The reaction mixtures were subjected to electrophoresis in 1% agarose gels (0.5 X TBE), and the DNA was stained with ethidium bromide (0.5 µg/ml) and imaged under UV light.

2.2.4. Cells and viruses

Vero E6 cells (kidney epithelial cells from the African green monkey, Chlorocebus aethiops) were obtained from the European Cell Culture Collection (ECACC) and maintained in Dulbecco modified Eagle's minimal essential medium (DMEM) supplemented with L-GlutaMAX, 10% heat-inactivated fetal bovine serum (FBS), 1 X nonessential amino acids, and 2 mM L-glutamine (all from Gibco, Life Technologies). All cell cultures were grown at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The Vero cell-adapted ASFV-Ba71V isolate was propagated as described previously (Carrascosa, Bustos, & de Leon, 2011), and infections were carried out at the indicated multiplicities of infection (MOI). At the end of the adsorption period (1 h), the inoculum was removed and the cells were washed twice with serum-free medium. Viral titers were determined by 50% tissue culture infectious dose ($TCID_{50}$) titration using Vero E6 cells and the Spearman-Kärber method (Kärber, 1931).

2.2.5. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from ASFV-infected Vero cells (MOI = 1.5) at different time points of infection, using an RNeasy minikit and an RNase-free DNase set (both from Qiagen) and following the manufacturer's protocols. First-strand cDNA was synthesized from 2 g total RNA using the Superscript II First Strand Synthesis System (Invitrogen) and analysed in duplicate by quantitative reverse transcription-PCR (qRT-PCR). The real-time PCR mixtures contained 1 µl (1:20) of template cDNA, 2.5 µl of forward and reverse primers (at 50 nM) (Table 7), 12.5 µl of Maxima SYBR green/ROX qPCR master mix (Thermo Scientific), and sterile water to a final volume of 25 µl per tube. The thermal-cycling conditions were initial denaturation at 95°C for 10 min and 40 cycles of 95°C for 10 s and 60°C for 60 s, followed by storage at 4°C for further use. qRT-PCR analysis was performed in a 7300 real-time PCR system (Applied Biosystems), and the mRNA levels of viral genes (A104R, CP204L, and B646L genes) and of the reference gene (cyclophilin A gene) were quantified using the standard curves of different plasmids (pGEMTeasy_A104R, pGEM-Teasy_CP204L, pGEM-Teasy_B646L, and pGEM-Teasy_Cyclophilin A). Only data from qRT-PCRs showing an amplification efficiency of ≥0.92 and an R^2 value of ≥0.98 were considered in the analysis.

Target	Primer name	Sequence Sense Sequence (5´- 3´)	Orientation
ASFV-A104R[a]	A104R_Fw	GGAATTCCATATGATGTCGACAAAAAAAAAGCCCACAATTA	Forward
ASFV-A104R[a]	A104R_Rev	TCCGCTCGAGATTTAACATATCATGAACAGGTTTCAATGC	Reverse
ASFV-A104R[a]	A104R_RT_Fwe	AGCGGCAGATACCCAGTTAA	Forward
ASFV-A104R[a]	A104R_RT_Fw	ACCCGGAATCAAGTTCACCG	Forward
ASFV-A104R[a]	A104R_RT_Rev	CGGCTTTATGTTCAGGCTTGG	Reverse
Cyclophilin A	Cyclo_Fw	AGACAAGGTTCCAAAGACAGCAG	Forward
Cyclophilin A	Cyclo_Rev	AGACTGAGTGGTTGGATGGCA	Reverse
Cyclophilin A	Cyclo_Fwe	TGCCATCCAACCACTCAGTCT	Forward
ASFV-B646L[a]	VP72_Fw	ACGGCGCCCTCTAAAGGT	Forward
ASFV-B646L[a]	VP72_Rev	CATGGTCAGCTTCAAACGTTTC	Reverse
ASFV-CP204L[a]	VP32_Fw	TGCACATCCTCCTTTGAAACAT	Forward
ASFV-CP204L[a]	VP32_Rev	TCTTTTGTGCAAGCATATACAGCTT	Reverse

Table 7. Primers used in the present study.
[a] Primers were designed based on the full genome sequence of ASFV-Ba71V isolate (GenBank/EBML, accession number: ASU18466).

2.2.6. Quantification of ASFV genomes by qPCR

Viral DNA was extracted from Ba71V-infected Vero cells (MOI = 0.1) transfected with siRNA targeting A104R or GAPDH (control group), at 72 hpi, using a High Pure Viral Nucleic Acid kit (Roche). The number of viral genomes was determined by quantitative PCR as described by King et al. (2003).

2.2.7. Antibodies

pA104R labelling was performed with a mouse polyclonal antiserum raised against pA104R using the purified recombinant protein (1:100). Young male mice (BALB/c; 4 to 6 weeks old) were injected subcutaneously with 100 µg of pA104R, purified as described above and combined with Freund's complete adjuvant. Following the primary injection, two booster injections were administered at 2-week intervals. Total blood specimens were collected 10 days after the final booster injection, and the sera were aliquoted and stored at 20°C until they were used for immunoblotting and immunofluorescence studies. The specificity of the polyclonal antiserum was tested against purified recombinant ASFV-pA104R and whole infected-cell extracts. The immunostaining of ASFV-infected cells was performed by incubation

with an in-house anti-ASFV polyclonal antibody (1:100) produced in swine. Two secondary fluorescence-conjugated antibodies were used as follows: anti-mouse fluorescein isothiocyanate (FITC) (1:300; sc-2099; Santa Cruz Biotechnology) and anti-swine Texas Red (1:500; ab6775; Abcam). For immunoblot analysis, three primary antibodies (anti-pA104R, 1:100; anti-α-tubulin, 1:1,250 [number 2125; Cell Signaling Technology]; anti-β-actin, 1:200 [SC-69879; Santa Cruz Biotechnology) and two HRP-conjugated secondary antibodies (anti-rabbit IgG, 1:20,000 [4010-05], and anti-mouse IgG, 1:32,500 [1010-05; both from Southern Biotech]) were used. All antibody dilutions were performed in blocking solution and incubated according to the manufacturers' recommendations.

2.2.8. Protein extraction and Western blotting

Vero cells grown in 6-well plates (5.6 X 10^4 cells/cm^2) were infected with the ASFV-Ba71V isolate (MOI =5) and, when indicated, exposed to 50 µg/ml AraC (Sigma-Aldrich) after the adsorption period (1 h). Before sampling, mock-infected, infected, and AraCtreated infected cells were washed twice with PBS and lysed in ice-cold modified RIPA buffer (25 mM Tris, pH 8.2, 150 mM NaCl, 0.5% [vol/vol] NP-40, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS) supplemented with protease inhibitor cocktail (Complete, Mini, EDTA free; Roche) and phosphatase inhibitor cocktail (PhosStop; Roche). Clarified whole-cell lysates harvested at 4, 8, 12, 14, 16, 18, and 20 hpi were further subjected to SDS-PAGE, using 8 to 16% (wt/vol) polyacrylamide separating gels, and transferred to a 0.2-µm-pore-diameter nitrocellulose membrane (Whatman; Schleider & Schuell) by electroblotting.

For Tx solubility analysis, after the wash step with PBS, a buffer containing 50 mM HEPES (pH 7.6), 100 mM NaCl, 2 mM EDTA, 250 mM sucrose, 0.1% Tx supplemented with protease (Complete, Mini, EDTA free; Roche) and phosphatase (PhosStop, Roche) inhibitors was added to the Vero cells. ASFV-infected and mock-infected cells were collected at 16 hpi, and after centrifugation (10,000 X g for 10 min at 4°C), the pellet containing Tx-insoluble proteins was lysed in RIPA buffer. Then, both Tx-soluble (supernatant) and Tx-insoluble fractions were analyzed in SDS-polyacrylamide (8 to 16% [wt/vol]) gel electrophoresis. The blot membranes were blocked with PBST containing 5% (wt/vol) BSA (Sigma-Aldrich) for 1 h at RT and further incubated with specific primary antibodies (1 h at RT). Then, the secondary antibodies conjugated with HRP were also incubated for 1 h at RT. All the antibody incubations were followed by three 10-min wash steps with PBST, and protein detection was performed using a chemiluminescence detection kit (Pierce ECL Western blotting substrate; Thermo Scientific) on Amersham Hyperfilm ECL (GE Healthcare). α-Tubulin and β-actin were used as loading controls.

2.2.9. Immunofluorescence studies

At the indicated time points, ASFV-infected Vero cells (MOI = 2) growing on glass coverslips in 24-well plates (5.0 X 10^4 cells/cm^2) were fixed with 3.7% (wt/vol) paraformaldehyde in HPEM buffer {25 mM HEPES, 60 mM PIPES [piperazineN,N'-bis(2-ethanesulfonic acid)], 10 mM EGTA, 1 mM $MgCl^2$} for 10 min and permeabilized with PBS–Triton X-100 (0.2%, [vol/vol]) for 5 min at RT. The cells were then washed with PBS, blocked with PBST and BSA (1% [wt/vol]) for 30 min, and incubated with primary antibodies for 1 h. All procedures were performed at RT, and all antibody incubations were performed in a dark humidified chamber to prevent fluorochrome bleaching. Vectashield mounting medium with DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories) was used to visualize the nucleus and the viral factories. Fluorescence images were acquired using a Leica DMIRE2 epifluorescence microscope equipped with a 40X objective and analysed with ImageJ open-source software (version IJ 1.48g; National Institutes of Health, Bethesda, MD, USA) and Adobe Photoshop CS5 software (Adobe Systems, Inc.).

2.2.10. ASFV-A104R downregulation by siRNA

Two siRNAs targeting A104R transcripts (Table 8) and a siRNA duplex targeting GAPDH (siRNA-GAPDH) (Silencer GAPDH siRNA Control; number 4605) were purchased from Ambion (Applied Biosystems). siRNA-A104R was designed based on the full genome of the ASFV-Ba71V isolate (GenBank/EBML accession number U18466.2). The transfection mixtures were prepared by adding siRNAs to Opti-MEM medium (Gibco, Life Technologies) and to HiPerFect transfection reagent (Qiagen), following the manufacturer's instructions. Before addition to the cell cultures, the mixtures were homogenized by pipetting and incubated for 30 min at RT to allow the formation of transfection complexes. Vero cells grown in 24-well plates (2.0 X 10^4 cells/cm^2) were transfected with siRNAs at 10 and 50 nM. After 8 h, the transfection complexes were removed and replaced with fresh DMEM supplemented with 10% FBS before infection (MOI = 0.1). Infection was allowed to proceed for 72 h. The efficacies of the siRNAs were evaluated by qRT-PCR, comparing the A104R mRNA levels between mock-transfected cells and cultures transfected with siRNA-A104R_1 or siRNA-A104R_2. The antiviral effects of siRNA-A104R_2 were evaluated by quantifying the ASFV genomes and the cytopathic effect (CPE) and by virus yields at 72 hpi. In addition, the mRNA levels of an early and a late viral gene (CP204L and B646L genes) were determined by qRT-PCR and compared with those of nontransfected infected cells at 8 and 16 hpi.

Target	Primer designation	Sequence (5´- 3´)
ASFV-A104R[a]	siRNA-A104R_1	ACAUAAAGCCGUAAAGAUAUUUU
ASFV-A104R[a]	siRNA-A104R_2	GCAGAUACCCAGUUAAAUAUUUU

Table 8. Sequences of siRNAs used to knockdown expression of pA104R in ASFV-infected Vero cells. [a] Primers were designed based on the full genome sequence of ASFV-Ba71V isolate (GenBank/EBML, accession number: ASU18466).

2.3. Results

2.3.1. pA104R forms distinct DNA-protein complexes in the presence of oligonucleotides with different length

Although previous studies reported that the ASFV ORF A104R-encoded protein has significant sequence homology with bacterial histone-like proteins and pA104R binds to both single- and double-stranded calf thymus DNA cellulose columns (Borca et al., 1996; Neilan et al., 1993), the DNA-binding activity of this protein remains poorly understood.

In order to evaluate pA104R DNA-binding affinity and the DNA binding site size, oligonucleotides with different lengths (from 10 to 50 nucleotides [nt]) were used in EMSA. Although no protein-DNA complexes were detected when the purified pA104R was incubated with very short oligonucleotides (10 nt) (data not shown), the viral protein was able to bind oligonucleotides with lengths of 20 to 50 nt (Fig. 9), revealing that the minimum DNA length required to elicit binding is between 11 and 20 nt. Additionally, three distinct protein-DNA complexes were identified when pA104R was incubated with ssDNA probes with a length of 40 nt or 50 nt (Fig. 9, arrows), whereas a single protein-DNA complex was detected when the reaction mixtures contained oligonucleotides of 20 or 30 nt (Fig. 9, asterisks), suggesting that the minimal DNA length required for pA104R binding is about 14 to 16 nt.

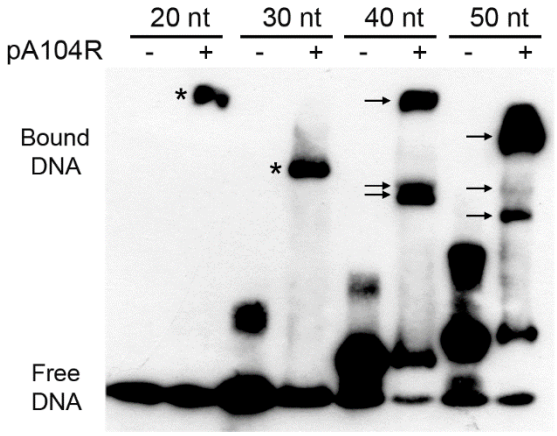

Figure 9. pA104R binds to DNA fragments with different lengths and shows a binding site size of about 14 to 16 nt.
EMSA was performed using 2.5 μM recombinant purified pA104R and 1 pmol of single-stranded oligonucleotides with increasing lengths (20, 30, 40, and 50 nt). After 30 min of incubation at room temperature, the reaction mixtures were subjected to native polyacrylamide gel electrophoresis, and biotin-labeled DNA was detected using a streptavidin-HRP antibody. The asterisks and arrows indicate bands corresponding to pA104R-DNA complexes. The unidentified DNA bands represent oligonucleotides annealed during incubation.

2.3.2. pA104R binds both ssDNA and dsDNA in a wide range of temperatures, pH, and salt concentrations and in an ATP-independent manner

To further characterize pA104R DNA-binding activity, increasing concentrations of protein (0.05, 0.25, 0.5, 1.25, 2.5, 3.75, and 5 μM) were incubated with 1 pmol of 5'-biotin-labeled 30-nucleotidelong ssDNA or dsDNA for 30 min at room temperature (RT). EMSA showed that pA104R binds both ssDNA and dsDNA, at minimum concentrations of 1.25 μM (Fig. 10A) and 0.5 μM (Fig. 10B), respectively, with the intensity of the free-DNA band decreasing with increasing concentrations of pA104R. Indeed, for protein concentrations above 0.5 μM, no free dsDNA was present, in contrast to the large amounts of ssDNA found even at 5 μM concentration, proving that pA104R has higher binding affinity to dsDNA than to ssDNA. A supershift assay was also performed to confirm the specific binding of pA104R to DNA. The addition of pA104R antibody to the binding reaction mixture resulted in the presence of a supershifted band with lower mobility than when the antibody was not added (Fig. 10C). Since the serum produced against purified pA104R specifically detected this protein by Western blotting (Fig. 10D), the observed supershifted band corresponded to pA104R antibody bound to the protein-ssDNA and protein-dsDNA complexes.

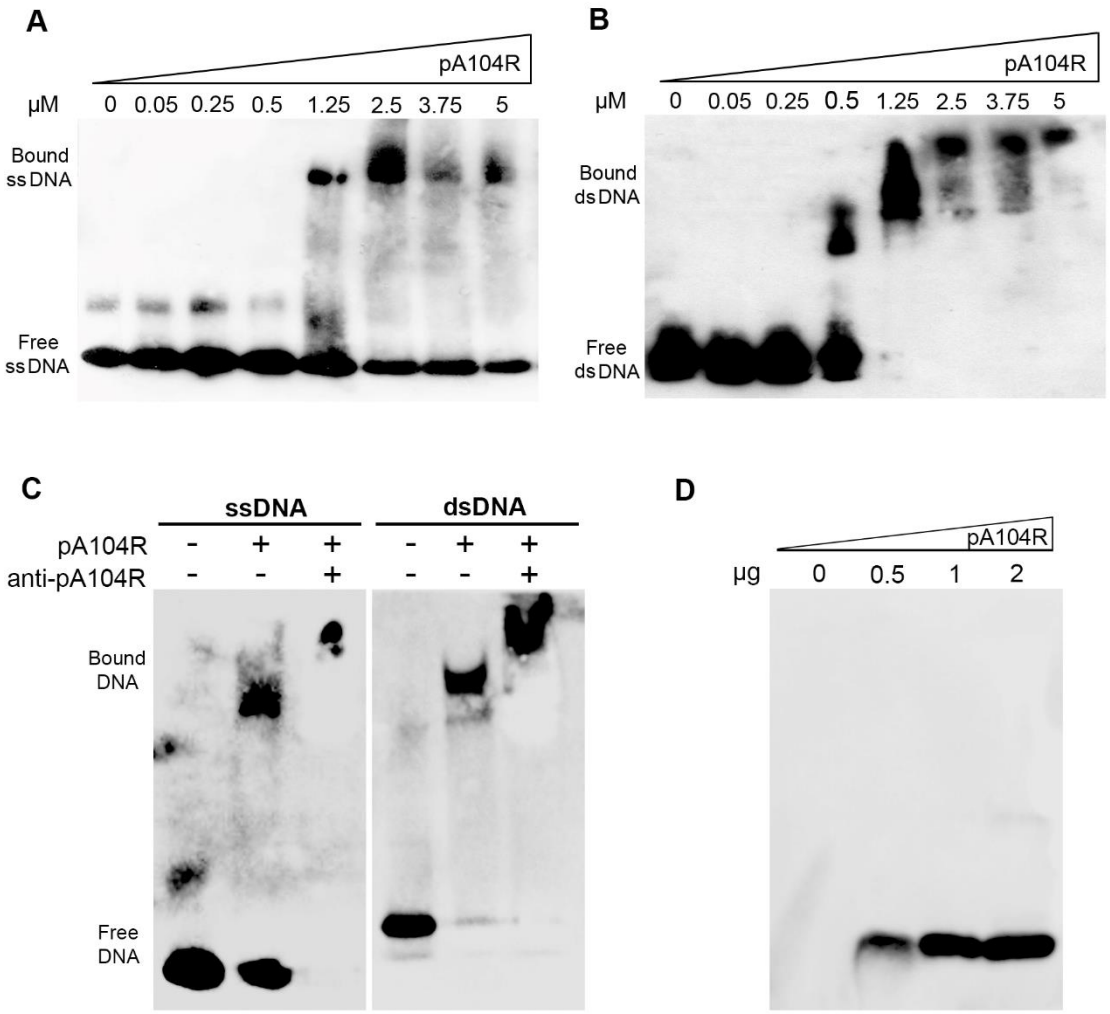

Figure 10. pA104R binds to dsDNA with higher affinity than to ssDNA.
(A and B) Reaction mixtures containing increased concentrations of recombinant purified ASFV pA104R and 1 pmol of biotin-labelled 30 nt ssDNA (A) or biotin-labelled 30 nt dsDNA (B) were incubated for 30 minutes at room temperature, followed by resolution on a native polyacrylamide gel and detection using a streptavidin-HRP antibody. (C) A supershift assay was performed by adding an anti-pA104R antibody to the binding mixture 5 min after the beginning of the reaction. (D) The specific recognition of the serum used was tested against the purified pA104R by Western blotting.

Although the formation of protein-DNA complexes was easily detected after a short incubation time (1 min), a larger number of complexes were formed with longer incubation times (Fig. 11A). EMSAs also revealed that the DNA-binding activity of pA104R was affected by the NaCl concentration and was higher at 0.1, 0.25, and 0.5 M; residual at 0 M and 1 M; and absent at 2 M (Fig. 11B). Furthermore, pA104R was able to bind DNA at a wide range of temperatures (4 to 37°C) (Fig. 11C) and pH values (Fig. 11D), with similar affinities and in an ATP-independent fashion (Fig. 11E). The pA104R binding site size remained constant under all experimental conditions.

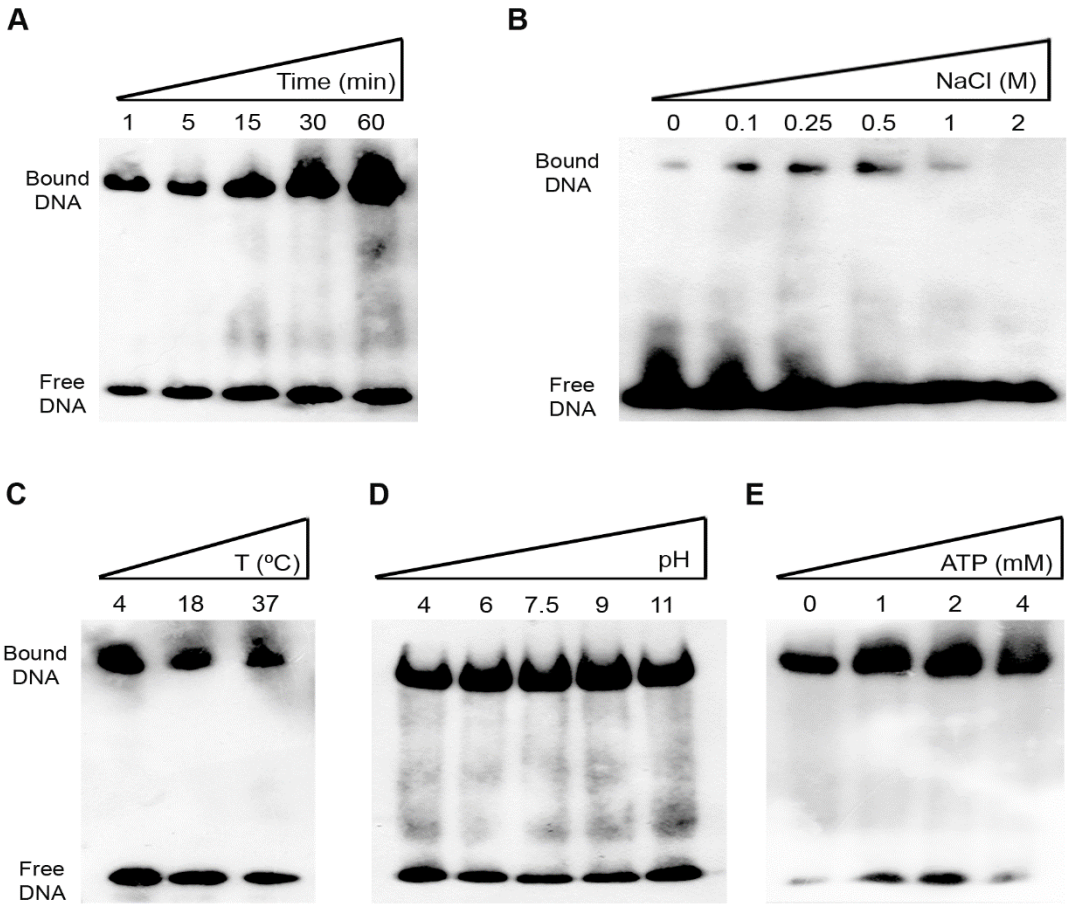

Figure 11. pA104R exhibits high DNA-binding affinity at a wide range of temperatures and pH values in an ATP-independent manner and is affected by ionic strength.
Binding reaction mixtures containing 2.5 µM of recombinant purified pA104R and 1 pmol of biotin-labelled 30 nt ssDNA were incubated for 30 min in a binding buffer. The incubation periods (A), NaCl concentrations (B), temperatures (C), pH values (D) and ATP concentrations (E) varied as indicated.

2.3.3. The arginine-69 residue is required for the DNA-binding activity of pA104R

Although arginine at position 69 and proline at position 74 of pA104R, both belonging to the DNA-binding domain, are conserved in all ASFV isolates and in bacterial histone-like proteins (Luscombe & Thornton, 2002), their roles in pA104R's DNA-binding activity are unknown. In order to evaluate if these residues are involved in protein-DNA interactions, three point mutants were generated by site-directed mutagenesis: pA104R^{R69A}, pA104R^{R69K}, and pA104R^{P74A}.
Our results showed that replacement of the positively charged arginine at position 69 (Arg^{69}) by a nonpolar amino acid (alanine) (pA104R^{R69A}) severely reduces DNA-binding activity (Fig. 12B) in comparison to the wild-type (wt) protein (pA104R^{wt}) (Fig. 12A). Interestingly, when Arg^{69} was replaced by another positively charged amino acid (lysine) (pA104R^{R69K}), the DNA-binding activity was not affected (Fig. 12C), suggesting that a positively charged residue at this position is critical for protein-DNA interactions. Finally, replacement of the proline at position 74 by an alanine residue (pA104R^{P74A}) did not significantly change the ability of pA104R to bind DNA (Fig. 12D).

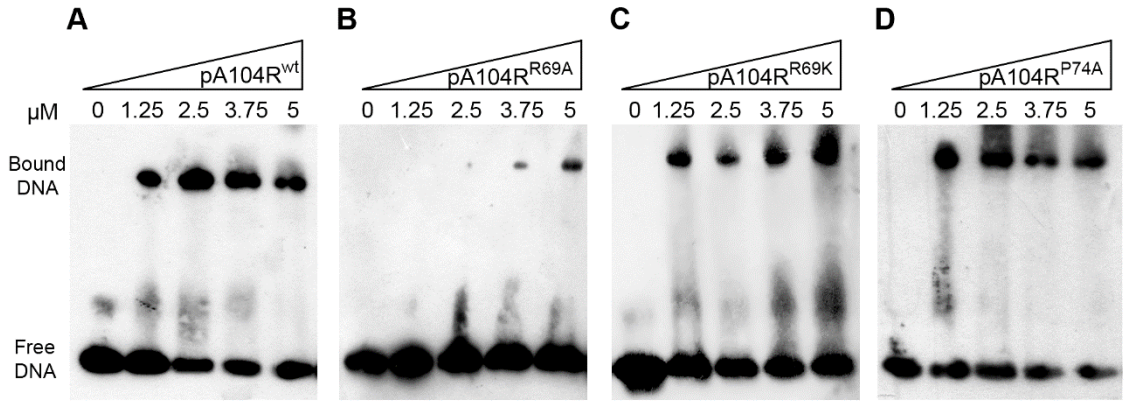

Figure 12. Arg^{69} is needed for the efficient pA104R DNA-binding activity, in contrast to the Pro^{74} residue. Wild-type pA104R (pA104R^{wt}) (A) and point-mutants at arginine residue 69 (pA104R^{R69A}, pA104R^{R69K}) (B and C) or at proline residue 74 (pA104R^{P74A}) (D) were added at increasing concentrations (1.25, 2.5, 3.75 and 5 μM) to 1 pmol of biotin-labelled 30 nt ssDNA and incubated for 30 min at room temperature.

2.3.4. pA104R cooperates with ASFV-topoisomerase II (pP1192R) to modulate DNA supercoiling

Considering that in bacteria, histone-like proteins and topoisomerases work together in spatial genome organization (Bensaid et al., 1996; Ghosh et al., 2014; Macvanin & Adhya, 2012; Malik et al., 1996), and knowing that ASFV also codes for a type II DNA topoisomerase (pP1192R), we aimed to investigate whether this phenomenon is maintained in ASFV. For this purpose, increasing concentrations of pA104R (0.01, 0.05, 0.1, 0.25, and 0.5 μM) were incubated with 150 ng of relaxed pBR322 DNA, in the presence or absence of ASFV topoisomerase II (0.01 μM), for 30 min at 37°C in a reaction volume of 20 μl. When pA104R was incubated alone with the relaxed plasmid, no supercoiling activity was detected. However, supercoiled DNA molecules were observed when pA104R, at high concentrations (0.25 and 0.5 μM), was added to a mixture of pP1192R and relaxed pBR322 DNA (Fig. 13). A similar, yet less pronounced, result was obtained when pA104R was added prior to the addition of pP1192R (data not shown).

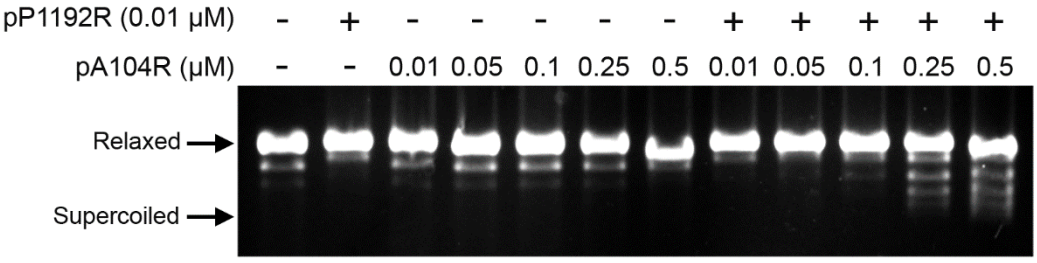

Figure 13. pA104R has DNA supercoiling activity in the presence of ASFV-topoisomerase II (pP1192R). pA104R and/or ASFV-topoisomerase II was incubated, at the indicated concentrations with 150 ng of relaxed pBR322 DNA for 30 minutes at 37 ºC and subjected to agarose gel electrophoresis. ASFV-topoisomerase II was added to incubation mixtures 10 min prior to pA104R addition.

2.3.5. The A104R gene encodes a late protein that localizes with viral DNA replication sites

Although low levels of A104R transcripts were detected from 2 h postinfection (hpi) onward, the transcription pattern of the gene closely resembles the transcriptional dynamics of the ASFV B646L late gene compared to the viral CP204L early gene. Indeed, the A104R gene is mainly transcribed from 8 hpi onward, showing a maximum peak at 16 hpi, and is transcribed at much lower levels than the above-mentioned genes encoding capsid proteins (Fig. 14A). The immunoblot analysis revealed that pA104R is expressed in ASFV-infected Vero cells from 12 hpi onward, reaching a maximum concentration peak at 18 to 20 hpi (Fig. 14B). pA104R was not detected in infected cells exposed to cytosine arabinoside (AraC), an inhibitor of viral DNA replication and consequently of late-phase transcription, further supporting the notion that pA104R is a late viral protein (Fig. 14B).

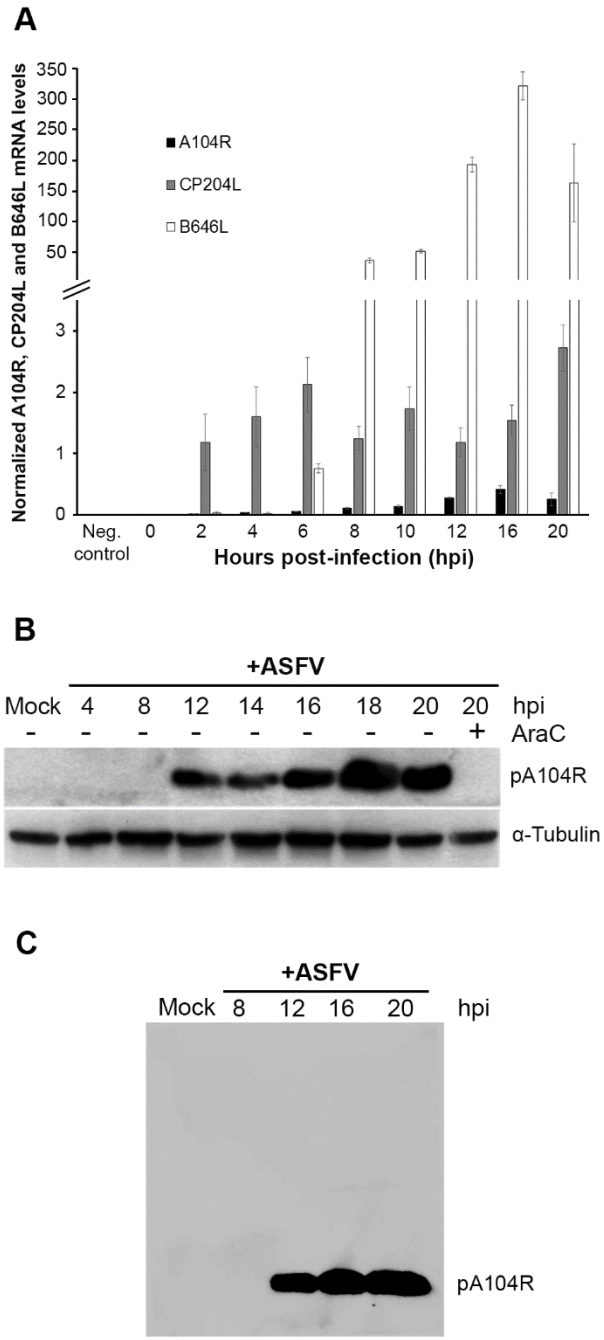

Figure 14. The A104R gene, encoding for a late protein, is transcribed from 2 hpi.
(A) A104R mRNA levels were measured by qRT-PCR at different time points after infection of Vero cells with ASFV-Ba71V isolate (MOI=1.5). Mock-infected cells were used as control. CP204L (vp32) and B646L (vp72) mRNA levels were determined in parallel, as controls for early and late viral gene expression, respectively. The results are shown as average ± standard error (SE) between the number of molecules of each viral gene and the number of molecules of the Cyclophilin A housekeeping gene. The results were obtained from two independent experiments run in duplicate. (B) Vero cells infected with Ba71V isolate (MOI=5) were harvested at the indicated time points. AraC (50 µg/ml) treatment was performed after the initial viral adsorption period (1 h), and cells collected at 20 hpi were lysed for the immunoblot assay. α-Tubulin was used as a loading control. (C) The specific recognition of the serum used was tested against whole infected-cell extracts by Western blotting.

Immunostaining studies revealed that pA104R shows a speckled nuclear distribution with nucleolar exclusion and accumulation in the cytoplasmic viral factories (Fig. 15A) from 12 hpi onward. The specificity of the serum used against whole infected-cell extracts is shown in Fig. 14C.

Taking into consideration the DNA-binding activity of pA104R and its subcellular localization in infected Vero cells, we aimed to evaluate if the viral protein is bound to the Triton X-100 (Tx)-insoluble elements (e.g., viral DNA, nuclear matrix, and cytoskeleton). The distribution of pA104R was investigated in Triton X-100 subfractions of whole infected-cell lysates harvested at 16 hpi. Remarkably, the polyclonal antibody raised against pA104R recognized only a 12-kDa band in the Triton X-100-insoluble fraction (Fig. 15B), indicating that pA104R is anchored to detergent-insoluble components/structures.

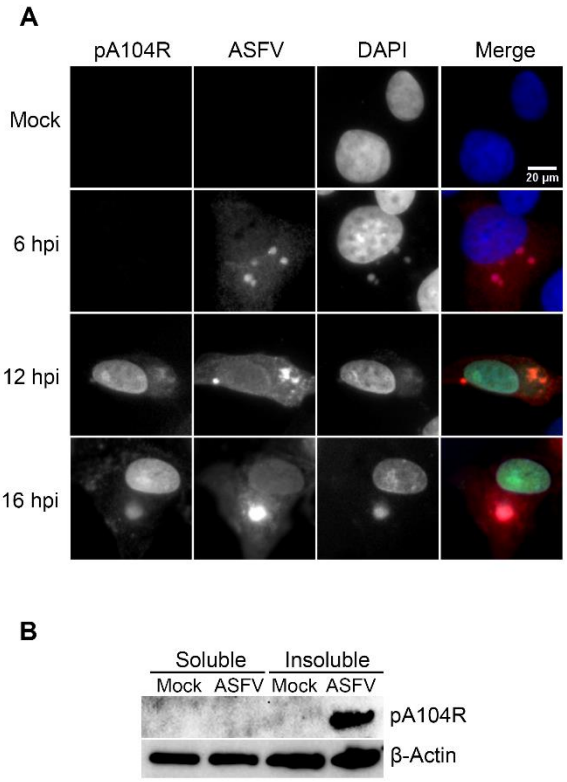

Figure 15. pA104R localizes within cell nuclei and cytoplasmic viral factories and is bound to Triton X-100-insoluble components/structures.
(A) Vero cells infected with the Ba71V isolate (MOI = 2) were fixed at 6, 12 and 16 hpi, and analysed by immunofluorescence for pA104R, ASFV and DNA. In the merged images, pA104R, ASFV and DAPI staining is shown in green, red and blue, respectively. Representative images of at least two independent experiments are shown. (B) For Triton X-100 fractionation procedure, Vero cells were resuspended in buffer containing 0.1% Tx-100 and fractionated.

2.3.6. Knockdown of pA104R reduces viral infection

To further explore the biological role of pA104R, Vero cells were transfected with two small interfering RNA (siRNA) sequences targeting A104R transcripts and then infected with the ASFV-Ba71V isolate. Taking into consideration that the siRNA-A104R_1 duplex did not reduce the A104R mRNA levels and that the siRNA-A104R_2 sequence leads to a reduction of around 27% at 16 hpi (Fig. 16), further siRNA studies were performed using this sequence.

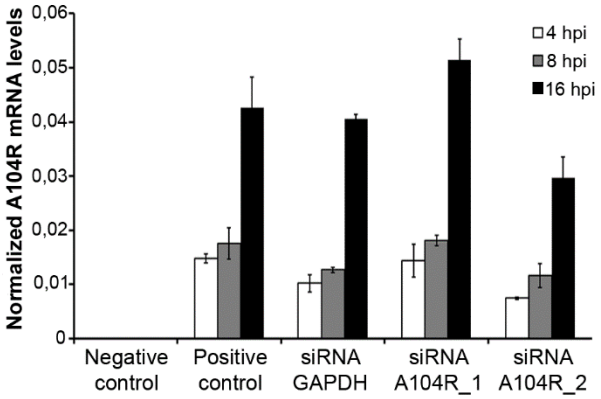

Figure 16. siRNA-A104R_2 reduces the mRNA levels of A104R by 27% at 16 hpi.
Vero cells were transfected with siRNA-A104R_1 (50 nM) or siRNA-A104R_2 (50 nM) for 8 hours and then infected with Ba71V (MOI = 0.1). A104R mRNA levels were quantified by qRT-PCR at 4, 8 and 16 hpi. Transfected noninfected cells were used as a negative control and nontransfected infected cells as a positive control. The results are shown as averages ± SE between the number of molecules of each viral gene and the number of molecules of the cyclophilin A housekeeping gene. The data were obtained from two independent experiments run in duplicate.

siRNA experiments revealed a lower number of viral progeny (− 82.0%) (Fig. 17A), a lower number of viral genomes (− 78.3%) (Fig. 17B), and reduced mRNA levels of a late ASFV gene that codes for the capsid protein p72 (B646L; − 47.6%) (Fig. 17C) in A104R-depleted cells compared with the control cells (transfected with siRNA-GAPDH [glyceraldehyde-3-phosphate dehydrogenase]). No differences in mRNA levels of CP204L, an early viral gene, were found between the groups (Fig. 17C).

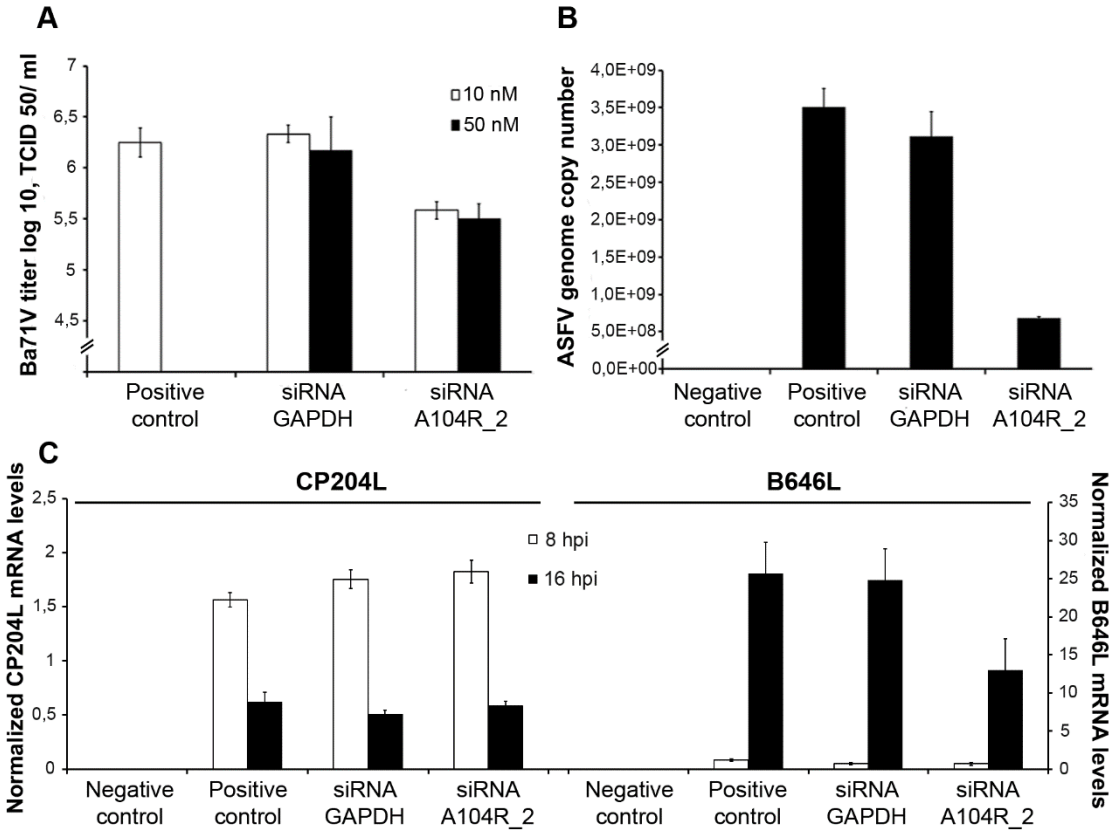

Figure 17. A104R mRNA knockdown inhibits ASFV infection.
(A) A reduction in viral yield (− 82.0%) was detected in ASFV-infected Vero cells (MOI = 0.1) transfected with siRNA-A104R_2 (10 and 50 nM), in comparison to controls (nontransfected infected cells and GAPDH siRNA-transfected infected cells) at 72 hpi. The virus yield of each supernatant was calculated from the average of three independent experiments. The error bars represent standard errors of the mean values. (B) A104R-depleted cells showed a decreased number of ASFV genomes (− 78.3%) compared to control cells at 72 hpi. The results were obtained from two independent experiments. (C) Decreased mRNA levels of B646L were observed in transfected infected cells (− 47.6%, 16 hpi) compared with the nontransfected infected cells and GAPDH siRNA-transfected infected cells. CP204L mRNA levels were not altered. The results are shown as averages ± SE between the number of molecules of each viral gene and the number of molecules of the cyclophilin A housekeeping gene. The data were obtained from two independent experiments run in duplicate.

2.4. Discussion

In this study, we found that purified recombinant pA104R binds to DNA, with higher affinity for dsDNA than for ssDNA, suggesting that at the cellular level, the protein is more capable of folding full-length ASFV genomes than intermediate single-stranded genomes. We also demonstrated that pA104R has a binding site size of around 14 to 16 nt and a minimal binding length of 11 to 20 nt, similar to those reported for other viral DNA-binding proteins (Loregian et al., 2007; Rochester & Traktman, 1998). The results obtained show that pA104R DNA-binding activity is stable at a wide range of temperatures (4°C to 37°C) and pH values (4 to 11), possibly to support ASFV replication in the soft tick vector (*Ornithodoros* spp.) and in swine. Indeed, ASFV replicates in the midgut epithelial cells of ticks at low pH values (Sojka et al., 2013) and enters swine macrophages by a low-pH-dependent endocytic pathway (Alonso et al., 2013). pA104R also binds DNA in an ATP-independent manner, suggesting that viral DNA packaging is a highly dynamic process, and a prompt DNA compaction may be

critical to generate a high number of ASFV particles and to maintain a successful infection. pA104R-DNA interactions were not detected under high-ionic-strength conditions (≥ 2 M NaCl), indicating that pA104R-binding activity involves ion pair formation, as reported for other viral DNA-binding proteins (Jiang, Komazin-Meredith, Tian, Coen, & Hwang, 2009; Komazin-Meredith et al., 2008; Wang et al., 2015).

Site-directed mutagenesis studies revealed that, as reported for other viral DNA-binding proteins (Chang & Miller, 2004; Paquet et al., 2014; Thain, Webster, Emery, Clarke, & Gaston, 1997), Arg^{69} is essential for the efficient DNA-binding activity of pA104R, probably due to its positive charge, since its replacement by the nonpolar amino acid alanine leads to reduction of pA104R activity (approximately 3-fold decrease) and its replacement by another positively charged amino acid (lysine) did not change the pA104R DNA-binding properties. In this study, a Pro^{74} residue could be replaced by a alanine without affecting the formation of pA104R-DNA complexes, indicating that van der Waals forces between Pro^{74} and nitrogenous bases are not essential for pA104R-DNA interactions, in contrast to previous findings for bacterial histone-like proteins (Luscombe, Laskowski, & Thornton, 2001; Luscombe & Thornton, 2002) and to *in silico* studies predicting that Pro^{74} is critical for pA104R's DNA-binding activity (Borca et al., 1996; Neilan et al., 1993).

Furthermore, we showed that pA104R has the ability to supercoil DNA in the presence of the recently characterized ASFV topoisomerase II (Coelho et al., 2016; Coelho, Martins, Ferreira, & Leitão, 2015; Freitas et al., 2016; Garcia-Beato et al., 1992b), as reported for some bacterial histone-like proteins (Bensaid et al., 1996; Ghosh et al., 2014; Kar et al., 2006; Luijsterburg et al., 2006; Malik et al., 1996; Rimsky & Travers, 2011; Swinger & Rice, 2004; Wang, 2002) and for some viral proteins involved in genome packaging (Bogner, Radsak, & Stinski, 1998; Borst et al., 2013; Ostapchuk, Yang, Auffarth, & Hearing, 2005; Thoma et al., 2006). The results of the supercoiling assay suggest that pA104R may participate in ASFV genome packaging, a rate-limiting step for virus maturation. Indeed, ASFV has to package its large DNA genome (170 to 190 kbp) into particles that have a diameter of only about 80 nm (Andrés, Simón-Mateo, & Viñuela, 1997), a very challenging event, since viral particles without DNA have been shown to be frequently released from infected cells by budding (Salas & Andrés, 2013). Although the mechanism by which ASFV packages its genome into a preassembled capsid remains unknown, this study points to the putative role of pA104R in promoting a stable, organized, and compact nucleoid, which is further supported by the observation that pA104R is localized over the central nucleoid structure (Borca et al., 1996; Salas & Andrés, 2013).

In vitro results from cell cultures revealed that the A104R gene is mainly transcribed during the late phase of infection, showing higher mRNA levels at 16 hpi, although in much smaller amounts than two ASFV structural genes (CP204L and B646L), indicating that pA104R is more likely to be involved in fundamental viral processes (e.g., DNA replication and transcription) than in capsid formation. At the protein level, pA104R was detected from 12 hpi onward, and

its expression was completely abrogated by AraC, confirming that pA104R is a late ASFV protein. The recruitment of pA104R to viral DNA replication sites (cytoplasmic factories) corroborates the results obtained with ASFV-infected macrophages (Borca et al., 1996) and strengthens the idea that the viral protein may participate in ASFV genome packaging. In parallel, its nuclear localization reinforces the concept that pA104R may also participate in the viral DNA replication that occurs inside this cellular compartment (Simões et al., 2015b) and/or in the heterochromatization of the cell genome (Simões et al., 2015a). The latter phenomenon could facilitate viral infection by silencing genes required to mount an immune response. Additionally, the nucleocytoplasmic distribution of pA104R is not mediated by the CRM1-dependent export pathway (data not shown). Finally, the partial depletion of A104R transcripts (− 27% at 16 hpi) reduces viral progeny release (− 82.0%), decreases the viral genome copy number (− 78.3%), and represses the transcription of a late viral gene (B646; 47.6%) without interfering with the transcription of the early viral gene CP204L.

Taken together, our results demonstrate that pA104R plays a key role in ASFV infection, probably by modulating the topological state of viral DNA and DNA-dependent events (e.g., DNA replication and transcription), as well as in viral genome packaging, suggesting that an ASFV mutant lacking ORF A104R may be a good strategy for the development of a vaccine against ASF. Indeed, an ASFV A104R-defective mutant would be expected to invade host cells and to express a number of immediate-early gene products, providing antigens that could induce a protective immune response in pigs and producing noninfectious progeny that undergo only one round of replication (e.g., aberrant/immature ASFV particles without DNA). Taking this into consideration, this study also highlights the importance of the establishment of a new complementing cell line that expresses pA104R in order to isolate and propagate an ASFV mutant lacking ORF A104R obtained by homologous recombination.

Acknowledgments

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CHAPTER III

Generation of a defective infectious single cycle African Swine Fever Virus particle lacking the A104R gene

Abstract

African swine fever (ASF) is a fatal viral disease of domestic swine and wild boar considered one of the main threats for pig husbandry. Despite the continuous efforts, using different strategies, no efficient vaccine, is so far, available to prevent the disease. Different defective infectious single cycle (DISC) or replication-defective mutants have been emerging as potential protective vaccines against different agents in response to ineffective viral vaccines using inactivated or live-attenuated viruses. In this study, we obtained an ASFV mutant lacking the A104R gene, describing for the first time the methodology to obtain an ASFV-DISC mutant. However, the recombinant mutant could not be isolated and further characterized, probably due to a non-complete complementation of the helper-cell line.

Keywords:

African swine fever virus, ASFV-pA104R, defective infectious single-cycle viral particle, vaccine.

3.1. Introduction

African swine fever (ASF) is a devastating disease, against which there is no available vaccine, mainly due to the complexity of the virus, the extend variation of African swine fever virus (ASFV) isolates and the technical difficulties involved in its development. The large gaps in knowledge concerning ASFV infection and immunity are also critical and further studies addressing this subject are mandatory to identify protective antigens.

Although the progress on an ASF vaccine will require an improvement of knowledge in ASFV biology and viral-host interactions (Rock, 2016), the vaccine development can indeed be obtained, as previous studies proved that pigs which recover from ASFV infection develop resistance to infection with the homologous virus (Boinas et al., 2004; Hamdy and Dardiri, 1984; Mebus and Dardiri, 1980).

Research work, developed along the last years, has shown that pigs are protected from challenge with related virulent isolates following infection, with natural low virulent isolates and with virus attenuated by passage in tissue culture or by deletion of genes involved in virulence (King et al., 2011; Lacasta et al., 2015; Leitão et al., 2001; Lewis et al., 2000; O'Donnell et al., 2015; Reis et al., 2016). However, the use of these live attenuated vaccines is still limited due to safety concerns and significant challenges will need to be faced before a vaccine becomes a reality.

In response to the unsuccessful classical viral vaccines (inactivated or live-attenuated viruses), a new class of vaccines has been developed to control other viral infections, such as the human immunodeficiency virus or the herpes simplex virus (Dudek & Knipe, 2006). Replication-defective and single-cycle defective mutant viruses of herpes simplex virus (HSV)

(Da Costa, Jones, & Knipe, 1999; van Lint, Torres-Lopez, & Knipe, 2007), HIV (Moussa et al., 2015), adenovirus (Crosby, Nehete, Sastry, & Barry, 2015), poxviruses (Coulibaly et al., 2005) and bluetongue virus (Matsuo et al., 2011) have been showing promising results as potential vaccines. These mutant viruses are defective for one or more functions essential to viral genome replication, viral protein synthesis or to the assembly of viral particles. This new strategy offers refreshing hopes for the control of important viral diseases. This methodology also has safety advantages when compared with the vaccines using inactivated virus, allowing the expression and the presentation of viral antigens to host MHC class I and II, resulting in immune responses with magnitude and durability equal to its replication-competent parental virus (Morrison & Knipe, 1996).

To the best of our knowledge, the generation of ASFV deletion mutants in essential genes, also called defective infectious single cycle particles (DISC), has never been explored to produce an efficient ASFV vaccine, mostly because the biologic role of viral proteins that are putatively involved in ASFV replication is still unknown. Previous results of our lab (Frouco, et al., 2017) strongly suggest that pA104R participates in the modulation of viral DNA topology and probably in genome packaging, and shows that the downregulation of this gene affects the transcription of other ASFV genes and induces a reduction of viral yields. Based in these findings, ASFV mutants lacking A104R gene may be used as a good strategy for the development of a vaccine against ASF, probably resulting on a DISC mutant that would express viral proteins during a first cycle of infection, though incapable to pursue a second cycle of infection once the A104R is predictable to be an essential gene for ASFV infection. Thus, it is expected that ASFV-A104R-DISC mutant would induce a strong and protective immune response in pigs, and could be used as a safe and efficient vaccine against ASF.

The methodology used to obtain an ASFV mutant lacking A104R gene is described in this study. In brief, a Vero cell line expressing the ASFV-A104R protein was established in our lab to propagate A104R-defective mutant; the plasmid vector was constructed to obtain the mutant by homologous recombination and recombinant mutants were generated. Due to technical difficulties, the isolation and further characterization of the recombinant virus was not achieved and therefore the biological function and essential role of the viral pA104R as essential steps to obtain a DISC vaccine against ASF could not be characterized.

3.2. Material and methods

3.2.1. Cells and viruses

Vero E6 cells (kidney epithelial cells of African green monkey *Cholorocebus sabaeus*) were obtained from the European Cell Culture Collection (ECACC), and maintained in DMEM (Dulbecco Modified Eagle's minimal essential medium) supplemented with L-Glutamax, 10% heat inactivated fetal bovine serum (FBS), 1X non-essential amino acids and 2mM L-glutamine (all from Gibco, Life Technologies). All cell cultures were grown at 37 ºC in a 5% CO_2 humidified atmosphere.

The Vero adapted ASFV-Ba71V isolate was previously subjected to serial limiting dilutions to ensure that virus suspension is homogenous and then was propagated as previously described (Carrascosa et al., 2011). Viral titres were determined by TCID50 (tissue culture infectious dose 50),calculation using Vero E6 and the Spearman-Kärber method (Kärber, 1931) and the infections were carried out at the indicated multiplicities of infection (MOI).

3.2.2. Construction of Vero-pA104R cell line

In order to obtain a cell line expressing ASFV-pA104R that allows the replication of defective virus on the expected essential A104R gene, Vero cells were lipofectamine-transfected with the linearized pIRES_ASFV-A104R vector, which was produced by inserting the open reading frame (ORF) of A104R into the vector pIRESneo (Clontech), kindly provided by Günther Keil (Friedrich-Loeffler-Institut, Germany). The ORF A104R was previously amplified by PCR from ASFV-Ba71V genomic DNA using the following primers: A104R_Fw GGA CTA GTA TGT CGA CAA AAA AAA AGC CCA CAA TTA and A104R_Rev TCC CCC GGG TTA ATT TAA CAT ATC ATG AAC AGG TTT CAA TGC. After transfection, cells with integrated copies of the expression vector were selected by the addition of 0.5 mg/ ml Geneticin® (G418 Sulphate, Gibco, Life Technologies). The surviving clones were expanded and a stable Vero-pA104R cell line was developed after 40 repeated passages by using antibiotic selective pressure (1 mg/ ml Geneticin®) (Fig. 18).

In order to verify if the ORF A104R was integrated in Vero-pA104R cell genome, the Vero-pA104R genomic DNA was extracted using Quick-gDNA™ Miniprep Plus Kit (Zymo Research), and the ORF A104R was detected by PCR.

Total RNA was also extracted using the RNeasy Mini Kit and RNase-free DNase Set (both from Qiagen) and following the manufacturer's protocols. First-strand cDNA was synthesized from the total RNA using the Superscript II First Strand Synthesis System (Invitrogen) and used as template for a nested-PCR detection of A104R transcripts. The expression of pA104R was confirmed by immunofluorescence and immunoblotting analysis as follow described.

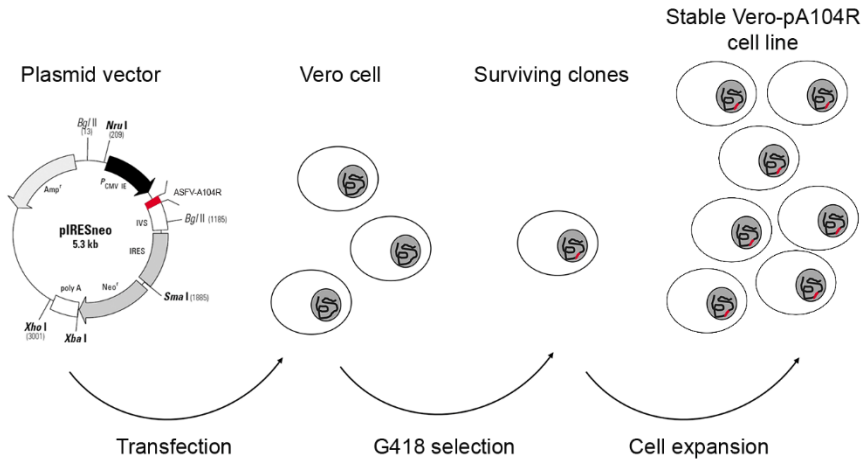

Figure 18. Schematic overview of construction of Vero-pA104R cell line.
Vero cells were transfected with the linearized pIRES_ASFV-A104R vector and then were subjected antibiotic selective pressure. The surviving clones were expanded and the Vero-pA104R cell line was stablished.

3.2.3. Immunofluorescence studies

Vero-pA104R cells growing on glass coverslips, in 24-well plates ($1.0x10^5$ cells), were fixed with 3.7% (w/v) paraformaldehyde in HPEM buffer (25 mM HEPES, 60 mM PIPES, 10 mM EGTA, 1 mM $MgCl_2$) for 10 minutes and permeabilized with PBS/Triton X-100 (0.2%, v/v) for 5 min at RT. Cells were then washed with PBS, blocked with PBST and BSA (1%, w/v) for 30 min and incubated with an anti-A104R polyclonal antibody (Frouco et al., 2017) for 1 hour. After the wash step with PBS, the secondary antibody anti-mouse Alexa Fluor 588 (Abcam) was also incubated for 1 hour. All procedures were performed at room temperature (RT) and all antibody incubations were performed in a dark humidified chamber to prevent fluorochrome bleaching. Vectashield mounting medium with DAPI (Vector Laboratories) was used to visualize the nucleus and the viral factories. Fluorescence images were acquired using a Leica DMIRE2 epifluorescence microscope equipped with a 40X objective and analysed with ImageJ open source software (version IJ 1.48g, National Institutes of Health, Bethesda, MD, USA) and Adobe Photoshop CS5 software (Adobe Systems, Inc.).

3.2.4. Protein extraction and western blotting analysis

After the wash step with PBS, a buffer containing 50 mM HEPES (pH 7.6), 100 mM NaCl, 2 mM EDTA, 250 mM sucrose, 0.1% Tx-100, supplemented with protease (cOmplete, Mini, EDTA-free from Roche) and phosphatase inhibitors (PhosStop, Roche) was added to the Vero-pA104R cells growing in 6-well plates ($5.0x10^5$ cells/cm^2). After a centrifugation step (10000 x g for 10 min, 4 ºC), the pellet containing Tx-insoluble proteins was lysed in RIPA buffer [25 mM Tris, pH 8.2, 150 mM NaCl, 0.5% (v/v) NP40, 0.5% (w/v) sodium deoxycolate, 0.1% (w/v) SDS]. Then, both Tx-soluble (supernatant) and Tx-insoluble fractions were

subjected to SDS-polyacrylamide (8-16%, w/v) gel electrophoresis and transferred to a 0.2 µm pore diameter nitrocellulose membrane (Whatman Schleider & Schuell) by electroblotting.

To obtain whole-cell protein extracts, ice-cold modified RIPA buffer supplemented with protease-inhibitor and phosphatase-inhibitor cocktail was added to the cells and also subjected to an electrophoresis analysis, as referred above.

Blot membranes were blocked with phosphate-buffered saline plus 0.05% (v/v) Tween-20 (PBST), containing 5% (w/v) of BSA (Sigma-Aldrich), for 1 hour at RT, and further incubated with an anti-A104R polyclonal antibody (Frouco et al., 2017) and an anti-β actin antibody (Santa Cruz Biothecnology) (1h, RT). Afterwards, the secondary antibody anti-mouse conjugated with HRP was also incubated for 1 hour at RT. All antibody incubations were followed by three 10-min wash steps with PBST and protein detection was performed using a chemiluminescence detection kit (Pierce® ECL Western Blotting Substrate, Thermo Scientific), on Amersham Hyperfilm ECL (GE Healthcare). β-Actin expression was used as loading control.

3.2.5. Construction of plasmid transfer vector

The plasmid transfer vector pΔA104R_GUS was constructed to support the deletion of the A104R gene from the genome of ASFV-Ba71V isolate. For this, a 500 bp fragment (L arm) located immediately upstream of the A104R gene at position 29666-30166 was amplified by PCR from Ba71V genomic DNA, using the Larm_Fw (GGT ACC ATT TTT TAT GCC ATG G) and Larm_Rev primers (TCT TGT ATA AAA AAC TAA ATA TTT TTT TAT AAA AAT ATT AAA TCT ATT AAA A). A 504 bp fragment (R arm) located downstream of the gene to be deleted at position 30478-30982 was also amplified using the PCR primers Rarm_Fw (GAT ATC TTA AAT AAT AAA GCC ATC ATC ATC G) and Rarm_Rev (TCT AGA AGT GAA TTT AGC GAG AAA T). In the 5´end of the right arm a restriction site was inserted to facilitate the insertion of the central region of the construct [B646L promotor and β-glucuronidase (Gus) gene]. The amplified L arm and R arm fragments were phosphorylated by T4 polynucleotide kinase (PNK, New England Biolabs) treatment during 30 minutes at 37 ºC, then were ligated with each other by T4 ligase (Thermo-Scientific), and the resulting fragment was cloned into the vector pJet1.2 (Thermo-Scientific). This vector was then digested with EcoRV (New England Biolabs) overnight at 37 ºC and dephosphorylated using Antarctic phosphatase (New England Biolabs) during 60 minutes at 37 ºC. The GUS gene from Escherichia coli, previously amplified using the PCR primers (GUS_Fw: ATT TAA TAA AAA CAA TAA ATT ATT TTT AT A ACA TTA TAT ATG TTA CGT CCT GTA GAA ACC CCA ACC; GUS_Rev: TCA TTG TTT GCC TCC CTG CTG) and phosphorylated, was then ligated into the cut plasmid resulting in the vector pΔA104R_GUS (Fig. 19). The pΔA104R_GUS vector included the GUS gene under the control of the B646L (p72) promoter flanked by adjacent regions of the A104R gene (L arm and R arm).

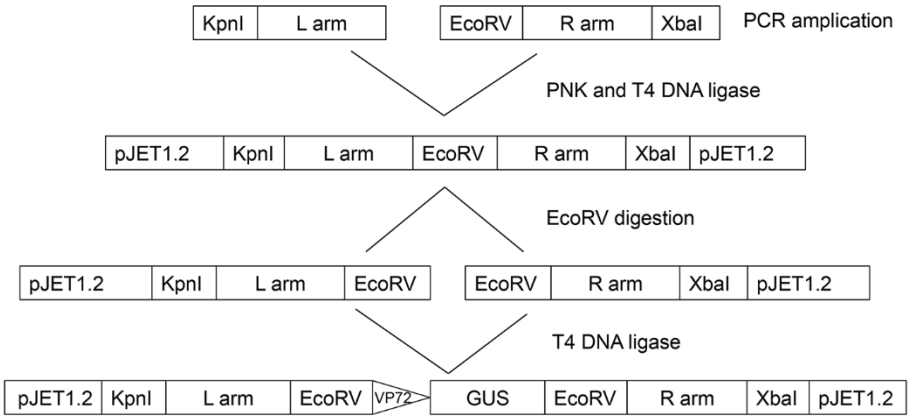

Figure 19. Schematic diagram showing the methodology used to construct the plasmid transfer vector. To facilitate the deletion of A104R gene from the genome of virus Ba71V, a plasmid transfer vector pΔA104R_GUS containing the B646L (p72) promoter and GUS gene flanked by adjacent regions of the A104R gene (L arm and R arm) was constructed.

3.2.6. Generation and purification of recombinant ASFV

The recombinant ΔA104R virus was obtained as described by Abrams and Dixon (2012) (Fig. 20). Briefly, Vero cells, growing in 24-well plates ($7.5x10^4$), were infected with ASFV-Ba71V isolate at a MOI of 10 and incubated at 37 ºC for 5h, and then the supernatant was removed and cells were washed with PBS (Gibco-Life Technologies). The transfection mixtures containing 200 µl Optimem (Gibco-Life Technologies), 2.5 µg pΔA104R_GUS vector and 5 µl TRANST-IT 2020 transfection reagent (Mirus) were homogenized, incubated for 30 minutes at RT and then added to the infected cells. After 4 hours of incubation, cells were supplemented with 400 µl of DMEM and the incubation continued at 37 ºC. The supernatant was collected at 48 hours post-infection (hpi). Additionally, another protocol described by Granja et al. (2004) in which Vero cells are first transfected and then infected was also tested in this study.

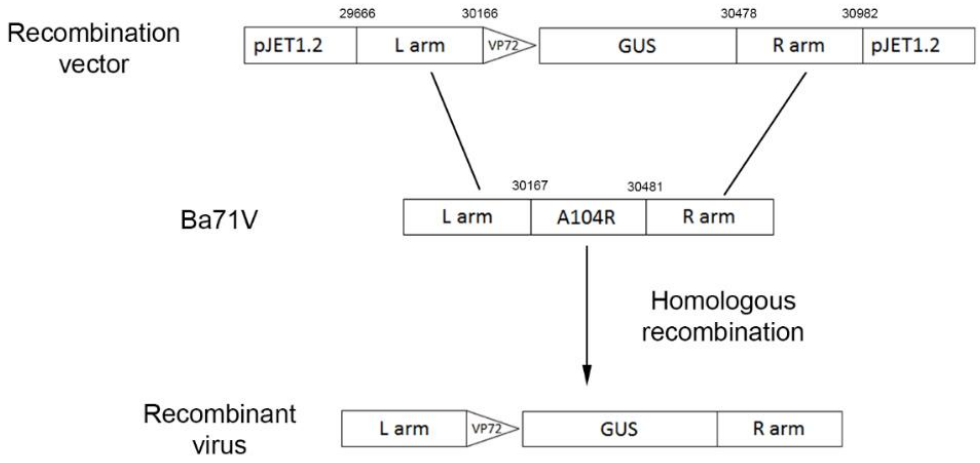

Figure 20. Schematic diagram representing the generation recombinant ASF viruses expressing GUS reporter gene.
Recombinant virus was obtained by homologous recombination between the left and right sequence regions (Larm and Rarm) of A104R gene on wild-type ASFV-Ba71V genome and recombination vector, resulting in the deletion of A104R gene and insertion of GUS gene under control of vp72 promotor.

Virus harvested from infected and transfected cells were used to infect new Vero cells on p6-well plates. Five hours after infection the virus inoculum was removed, the cells were washed with PBS and a 1% Agarose/ DMEM overlay containing 5% FBS (all from Gibco-Life Technologies) and 100 μg/ml X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid, Sigma-Aldrich) was used to cover the cells. The recombinant viruses were identified by the blue appearance of the plaques. Individual blue plaques were picked, homogenized in DMEM, used to infect a new cell layer and subjected to an additional plaque purifications.

Due to the technical difficulties and in order to obtain a purified recombinant virus, infection at limiting dilutions was also tested in Vero cells (p96-wells plates, $5x10^3$/ well). After 48 hours, 100 μg/ml X-Gluc was added to the cells and, similar to the plaques method, wells containing recombinant GUS expressing viruses acquired the blue appearance.

3.3. Results

3.3.1. A stable Vero cell line expressing ASFV-pA104R was established

The establishment of a helper cell-line, expressing the viral protein encoded by gene which is intended to be deleted, is mandatory to obtain and isolate a viral deletion mutant on an essential gene.

The helper cell-line supporting the deletion ASFV mutant on the A104R gene was successfully established in this study. The integration of the ORF A104R in Vero cell genome after the antibiotic selective pressure was positively confirmed by PCR (Fig. 21).

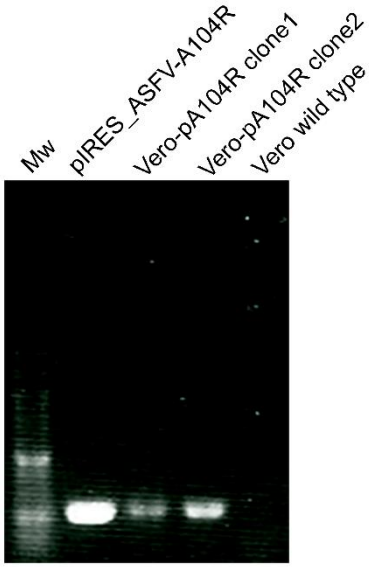

Figure 21. ORF A104R is successfully integrated in transfected Vero cell genome.
ORF A104R was amplified by PCR from the genome of Vero-pA104R clones which survive to G418 selection.

Immunofluorescence and immunoblotting studies were also performed to evaluate the expression of pA104R in the transformed cell line. Although the viral histone-like protein was detected diffusely distributed in cytoplasm and in some nucleus of Vero-pA104R (Fig. 22A), its presence could not be observed in western blot analysis. With this approach, the pA104R was only observed in Tx-insoluble protein fraction, showing a band with lower intensity than infected cells (Fig. 22B).

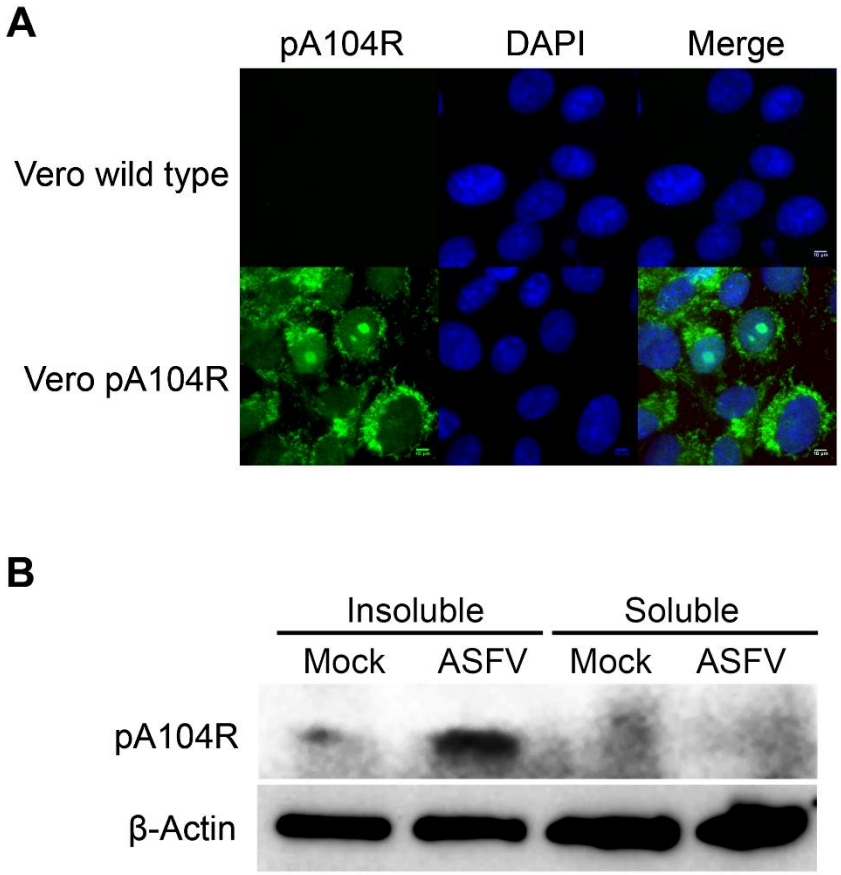

Figure 22. Complementary Vero cell line is expressing viral pA104R.
(A) Immunofluorescence studies showed the presence of ASFV-pA104R in the cytoplasm and nucleus of Vero-pA104R cells. pA104R and DAPI staining is shown in green and blue, respectively. (B) Transformed Vero cells non-infected and infected with Ba71V isolate (MOI=2; 18 hpi) were resuspended into buffer containing 0.1% Tx-100 and fractionated. pA104R was only detected in the Triton X-100-insoluble fraction, as expected, of non-infected and infected cells. B-actin was used as loading control.

3.3.2. Vero-pA104R cell line supports ASFV infection although lower titres are obtained

After being validated for the expression of pA104R, the obtained cell line was tested for its ability to allow infection by the ASFV-Ba71V isolate. Although cytopathic effect was observed in infected Vero-pA104R cells, lower viral progeny production was observed when compared to infected wild type Vero cells at 72 hpi (data not shown).

3.3.3. Deletion of the A104R gene from the Ba71V-ASFV isolate

Recombinant ASFV was constructed by homologous recombination between the left and right homology arms flanking the deletion site (L arm and R arm) of the recombination vector pΔA104R_GUS and the genome of Ba71V-ASFV isolate, within infected Vero cells expressing the viral pA104R. These cells were infected with ASFV-Ba71V isolate prior to the transfection with the pΔA104R_GUS vector, and the supernatants were collected after 48 hpi. When in the presence of X-Gluc (100 µg/ml), the supernatants from the recombination step acquired blue color, proving that the homologous recombination occurred and recombinant viruses expressing GUS were successfully obtained.

Viral progeny was quantified by current titration on complementary Vero-pA104R cells. Titers of recombinant virus were always much lower when compared with titers of the wild type virus (data not shown).

Transfection of infected cells was repeated using different concentrations of plasmid and transfection agent and different multiplicities of viral infection. Additionally another protocol used by Granja et al. (2004) was tested. However, regardless of the different procedures used, the difference between viral yields from wild type ASFV and recombinant virus remained high (data not shown).

To isolate recombinant ASFV-ΔA104R mutant, supernatants obtained from recombination steps containing wild type and recombinant virus were used to infect new Vero-pA104R cell cultures, which were afterwards covered with agarose containing X-Gluc. After 72 hpi, some blue lytic plaques were found, suggesting a productive cell infection by the recombinant virus mutants. Those plaques were collected, homogenized and passaged in new cell cultures. Considering that in the third passage no blue plaques were detected (Fig. 23), a different isolation approach was conducted in cell cultures in 96 well plates, using various dilutions of the supernatants from the recombination assays (Fig. 24). Using this method, blue-colored cells (expressing β-glucuronidase) were observed up to the third passage of the initial supernatant stock.

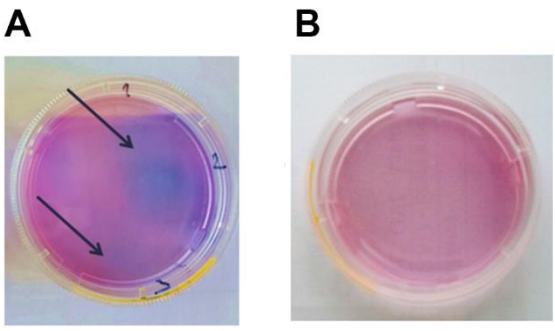

Figure 23. Isolation of recombinant virus using the plaques method.
Supernatants of the recombination step were used to infect new Vero-cells expressing pA104R, which were covered by agarose with X-Gluc **(A).** The blue plaques formed were picked and used to infect new cell cultures. No blue plaques were visible in a third passage **(B)**.

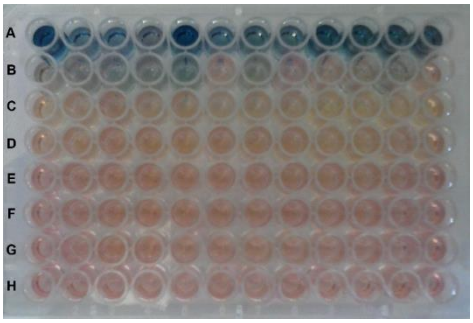

Figure 24. Isolation of recombinant virus using serial dilutions.
Supernatants of the recombination step were diluted [1:10 – 1:100000; **(A – E)**] in DMEM with X-Gluc and used to infect new Vero-cells expressing pA104R. The blue-colored supernatants were collected, diluted and used to infect a new cels. **(F – H)** Negative control.

Using the above-mentioned procedures, the isolation of the recombinant virus (ASFV-A104R) was not achieved after the third passage, and the successful replication of these recombinants in the established Vero-cell line expressing pA104R could not be confirmed.

3.4. Discussion

Traditionally, live-attenuated ASF viruses have been studied as vaccines against ASF. Moreover, pigs infected by low virulent ASFV isolates, attenuated virus by passages in tissue cultures or by deleted virus in genes involved in virulence, develop resistance to the homologous virus, but rarely to heterologous isolates (King et al., 2011; Lacasta et al., 2015; Leitão et al., 2001; Lewis et al., 2000; O'Donnell et al., 2015; Reis et al., 2016).

Nowadays, the unsuccessful efforts to develop an effective vaccine against ASF have been associated with lack of knowledge on different aspects of ASFV infection and immunity mechanisms (Rock, 2016). In order to better characterize ASFV biology and to identify viral antigens that could induce a protective immune response in the pig, different ASFV proteins putatively involved in viral replication and transcription, namely pA104R have been studied in our lab. This protein, also known as histone-like protein, has an extensive sequence similarity to bacterial proteins that are implicated in genome replication and packaging (Borca et al., 1996; Neilan et al., 1993). *In vitro* studies performed in our lab, showed that pA104R together with the ASFV-topoisomerase II (pP1192R) display DNA supercoiling activity, suggesting that pA104R participates in the modulation of viral DNA topology and may be involved in viral DNA replication, transcription and packaging (Frouco et al., 2017). These results emphasize that the A104R gene may constitute a good target for deletion to be used on a potential strategy to develop a DISC vaccine against ASF. Furthermore, the siRNA-A104R assays performed demonstrated that this late viral protein is essential to ASFV infection, thus a A104R-defective ASFV mutant is expected to enter host cell, not being able to condense the virus genome, resulting in aberrant/immature ASFV particles, non-infectious and incapable to produce a second cycle of infection.

Consequently, a vaccine development based on this strategy is expected to be safe, while viral antigens produced during the first cycle of infection can induce a protective immune response *in vivo*.

Taking these aspects into consideration, we proposed to generate an ASFV mutant lacking the ORF A104R. First, a complementing Vero cell line expressing pA104R was established, however, lower expression levels of the protein were detected in comparison to Vero cells infected with ASFV-Ba71V isolate. The establishment of a helper cell line is crucial to isolate and propagate a viral mutant lacking an essential gene for virus replication, assembly and morphogenesis. The recombinant mutants were successfully obtained by homologous recombination, as demonstrated by the blue appearance of the supernatant from infected and pΔA104R_GUS-transfected cells, when in the presence of X-Gluc. However, in the isolation process of ASFV-ΔA104R using either the plaque method or the limiting dilution, after three passages of blue-coloured plaques or supernatant, respectively, no recombinant mutants were detected, contrasting with the presence of wild type virus disclosed by observation of the cytopathic effect in non-blue-coloured cells.

Overall, our results suggest that the Vero-cell line expressing pA104R may not fully support the replication of recombinant virus, elucidating why no blue-plaques were observed after a third passage. Indeed, the low pA104R expression in these cells, and/ or other unknown factors (e.g. incorrect folding of the protein) may not allow the replication of recombinant virus.

The presence of blue-stained cells at early steps of the isolation process may be justified by one of two hypotheses: the recombinant virus is able to establish a first cycle of infection and the expression of GUS is enough to allow detection of blue-coloured cells; or, since wild-type ASFV is present at higher concentrations in the early steps of isolation, it is possible that these viral particles co-infect the same target cells as the recombinant virus, “providing” pA104R and “allowing” the replication of the recombinant virus, a mechanism described in other viruses (Frensing, 2015).

Although the ASFV-ΔA104R mutant could not be isolated, propagated and further characterized as a potential vaccine against ASF, the work here described opens new insights for a new strategy to obtain an efficient vaccine against ASF, reinforced by the fact that a replication-defective or a single-cycle mutant virus may constitute an invigorating approach to control ASF taking in consideration safety issues regarding vaccine development.

Acknowledgments

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The authors are grateful to Doctor Günther Keil (Friedrich-Loeffler-Institut, Germany) for providing pIRESneo plasmid and to Doctor Margarida Simões (Faculdade Medicina Veterinária, Lisboa) for her work in the development of the complementary cell line.

CHAPTER IV

Sodium phenylbutyrate abrogates African swine fever virus replication by disrupting the virus-induced hypoacetylation status of histone H3K9/K14

Gonçalo Frouco, Ferdinando B. Freitas, Carlos Martins and Fernando Ferreira. (2017). *Virus Research,* 242, 24-29.

Abstract

African swine fever virus (ASFV) causes a highly lethal disease in swine for which neither a vaccine nor treatment are available. Recently, a new class of drugs that inhibit histone deacetylases enzymes (HDACs) has received an increasing interest as antiviral agents.

Considering studies by others showing that valproic acid, an HDAC inhibitor (HDACi), blocks the replication of enveloped viruses and that ASFV regulates the epigenetic status of the host cell by promoting heterochromatinization and recruitment of class I HDACs to viral cytoplasmic factories, the antiviral activity of four HDACi against ASFV was evaluated in this study. Results showed that the sodium phenylbutyrate fully abrogates the ASFV replication, whereas the valproic acid leads to a significant reduction of viral progeny at 48 hours post-infection (-73.9%, p=0.046), as the two pan-HDAC inhibitors tested (Trichostatin A: -82.2%, p=0.043; Vorinostat: 73.9%, p=0.043). Further evaluation showed that protective effects of NaPB are dose-dependent, interfering with the expression of late viral genes and reversing the ASFV-induced histone H3 lysine 9 and 14 (H3K9K14) hypoacetylation status, compatible to an open chromatin state and possibly enabling the expression of host genes non-beneficial to infection progression. Additionally, a synergic antiviral effect was detected when NaPB is combined with an ASFV-topoisomerase II poison (Enrofloxacin).

Altogether, our results strongly suggest that cellular HDACs are involved in the establishment of ASFV infection and emphasize that further in vivo studies are needed to better understand the antiviral activity of HDAC inhibitors.

4.1. Introduction

African swine fever virus (ASFV) is the only member of the *Asfarviridae* family, sharing genomic and structure features with other eukaryotic viruses (e.g. poxviruses and iridoviruses), all belong to the nucleocytoplasmic large DNA viruses (NCLDV) clade (King et al., 2012). It causes a highly lethal disease in swine and is considered one of the most threatening diseases affecting pig husbandry, since neither a vaccine nor an antiviral treatment are available to control ASFV infection. Originally endemic in most of Sub-Saharan African countries, it was introduced in Transcaucasia (Georgia, Armenia, Azerbaijan) and in the Russian Federation during 2007, where it is maintained (Sánchez-Vizcaíno et al., 2013). More recently, new outbreaks have been reported in Ukraine (2012, 2014 and 2015), Belarus (2013), Lithuania, Estonia, Latvia and Poland (2014 and 2015) and more recently (2016) in Moldova (Gallardo et al., 2015).

To establish a successful infection, ASFV must overcome several host defence mechanisms, such as apoptosis, inflammatory and immune responses (Correia, Ventura, & Parkhouse, 2013; Galindo, Hernaez, Díaz-Gil, Escribano, & Alonso, 2008; Gil, Sepúlveda, Albina, Leitão, & Martins, 2008; Granja et al., 2006; Hurtado et al., 2004; Nogal et al., 2001) and, like other

viruses, ASFV may alter the epigenetic status of the host chromatin in order to control cellular gene expression to its own benefit (Chiocca et al., 2002; de Souza, Iyer, & Aravind, 2010; Knipe et al., 2013; Placek & Berger, 2010; Punga & Akusjärvi, 2000; Valls et al., 2007; Valls, de la Cruz, & Martínez-Balbás, 2003). Although the mechanisms by which viruses elicit this epigenetic reprogramming are not fully understood, their interaction with cellular Histone Deacetylases (HDACs) and Histone Acetyltransferases (HATs) are known to be critical for disrupting the host gene-expression program. Indeed, while HDACs catalyse the removal of the acetyl group from lysines, re-establishing the positive charge on the histones, thus inducing heterochromatinization and gene silencing (Dekker & Haisma, 2009), HATs promote the transfer of an acetyl group from a molecule of Acetyl Coenzyme-A onto the target lysine residue (Herbein & Wendling, 2010; Yang & Seto, 2007). In eukaryotic cells, a significant correlation was found between high acetylation levels of H3K9/K14 and euchromatin formation (an open chromatin state and transcriptionally active), contrasting with low acetylation levels which are associated with a tightly packed and silenced heterochromatin (Zaratiegui, Irvine, & Martienssen, 2007). Recently, eighteen HDACs were identified in mammals and classified as: Class I (HDAC 1, 2, 3 and 8), Class II (subdivided in subgroup IIa - HDAC 4, 5, 7 and 9, and IIb – HDAC 6 and 10), Class III (sirtuins) and Class IV (HDAC 11) (Bolden et al., 2006; de Ruijter et al., 2003). Remarkably, the enzymatic activity of class I and II HDACs can be pharmacologically modulated by inhibitors (HDACi), which have shown potent anti-parasitic (Bougdour et al., 2009; Chaal et al., 2010) and anti-tumour effects (Ahn, Ahn, & Yoon, 2011; Zimmerman et al., 2011). Some of these HDACi are also promising antiviral agents since they activate latent HIV, Epstein-Barr Virus and human cytomegalovirus, depleting reservoirs of persistent, quiescent infection (Archin et al., 2012; Ghosh et al., 2012; Huber et al., 2011; Michaelis et al., 2005; Radkov et al., 1999), and reduce replication of enveloped viruses by interfering with the stability of its particles (Vázquez-Calvo et al., 2011).

Considering the above data and knowing that ASFV modifies the epigenetic state of the host chromatin, promoting heterochromatinization of nucleus and recruiting HDAC1, HDAC2 and HDAC3 to viral factories (Simões et al., 2015a), we aimed to evaluate the antiviral activity of four HDAC inhibitors: Trichostatin A (TSA), Vorinostat (SAHA), valproic acid (VPA) and sodium phenylbutyrate (NaPB). Since, high acetylation levels of H3K9/K14 (H3K9/K14Ac) is an epigenetic marker for chromosome decondensation and a tell-tale sign of active transcription, we evaluate the dynamics of H3K9/K14 acetylation during ASFV infection and the effect of NaPB in the viral-induced H3K9/K14Ac status.

Finally, taking advantage of previous studies that identified a synergistic effect between HDACi and Topoisomerase's poisons on cancer treatment (Bevins & Zimmer, 2005; Marchion, Bicaku, Daud, Sullivan, & Munster, 2005a, 2005b), the antiviral activity of the NaPB was investigated in the presence of enrofloxacin, an ASFV-topoisomerase II poison (Freitas et al., 2016; Mottola et al., 2013). The results showed that all HDACi inhibit *in vitro* ASFV infection with NaPB

completely abrogated viral replication, at non-cytotoxic concentrations and in a dose-dependent manner. Our data also suggest that antiviral effects of NaPB resulted from the drug-induced higher H3 acetylated levels at K9/K14 residues, followed by an inhibition of the late viral protein synthesis and not interfering with the stability of the viral particles before cell entry. Finally, a synergistic antiviral effect was detected when NaPB was combined with enrofloxacin.

4.2. Material and methods

4.2.1. Cells and viruses

Vero E6 cells (kidney epithelial cells of African green monkey *Cholorocebus sabaeus*) were obtained from the European Cell Culture Collection (ECACC), and maintained in DMEM (Dulbecco Modified Eagle's minimal essential medium) supplemented with L-Glutamax, 10% heat inactivated fetal bovine serum (FBS), 1X non-essential amino acids and 2mM L-glutamine (all from Gibco, Life Technologies). All cell cultures were grown at 37 ºC in a 5% CO_2 humidified atmosphere.

The Vero adapted ASFV-Ba71V isolate was propagated as previously described (Carrascosa et al., 2011), and viral titers were determined by TCID50 (tissue culture infectious dose 50) titration using Vero E6 and the Spearman-Kärber method (Kärber, 1931).

4.2.2. Drugs and cytotoxic assay

Trichostatin A (TSA) and vorinostat (SAHA) were dissolved in DMSO, while valproic acid (VPA) and sodium phenylbutyrate (NaPB) were dissolved in water. Enrofloxacin was dissolved in NaOH at 0.1M. All drugs were purchased from Sigma-Aldrich and conserved according to the manufacturer´s recommendations.

Deleterious effects of HDAC inhibitors on cell viability were assessed by trypan blue dye exclusion assay, as previously described (Strober, 2001). Briefly, different concentrations of each drug were added, during 72 hours, to $1.0x10^4$ cells/cm^2 seeded in 24-well plates. Then, a representative sample of cells from supernatant and adherent monolayer was collected and diluted 1:1 in trypan blue solution (0.4%), with dead cells staining in blue, in contrast to unstained viable cells. To improve assay accuracy, at least 200 cells were analysed in three independent experiments. The cell viability values were normalized against the untreated control group, which was a value of 100%.

4.2.3. Drug treatment and viral infection

Vero cells were grown in 24-well plates, $1.0x10^4$ cells/cm^2, and treated with NaPB (5 mM), VPA (25 mM), TSA (100 nM) and SAHA (1 µM), 12 h before ASFV-Ba71V infection, which was carried out at a MOI of 0.1. At the end of the adsorption period (1h), the inoculum was removed and cells were washed twice with serum-free medium. Fresh medium was added containing

HDACi in the above concentrations and combined or not with enrofloxacin at 25µg/m and, this time point was considered as 0 h post-infection (hpi).
After cytopathic effect (CPE) observation, infected cultures were subjected to three freeze-thaw cycles and viral yields were determined by plaque assay (Kärber, 1931) at 24 and 48 hpi.

4.2.4. Antibodies

For immunoblotting analysis, two primary antibodies (anti-α-tubulin, # 2125, 1:200; anti-acetyl histone H3K9/K14, # 9677, 1:100) from Cell Signalling Technology and two HRP-conjugated secondary antibodies (anti-rabbit IgG, 4010-05, 1:100.000 from SouthernBiotech; anti-swine IgG, 114-035-003, 1:100.000 from Jackson ImmunoResearch Lab.) were used. For viral protein detection, an in-house produced swine anti-ASFV serum was used (1:200).
All antibody dilutions were performed in blocking solution (PBS-Tween 20 0.05%, v/v) and BSA (1%, w/v, Sigma-Aldrich, St. Louis, USA) and incubated according to the manufacturers' recommendations.

4.2.5. Direct immunofluorescence studies

Vero cells treated with NaPB and infected (MOI of 0.1), grown on glass coverslips, in 24-well plates ($5.0x10^4$ cells/cm^2), were fixed at 24 and 48 hpi, with 3.7% (w/v) paraformaldehyde in HPEM buffer (25 mM HEPES, 60 mM PIPES, 10 mM EGTA, 1 mM $MgCl_2$), during 10 minutes and permeabilized with PBS/Triton X-100 (0.1%, v/v) for 2 min at room temperature (RT). Then, cells were washed in PBS (Gibco, Life Technologies), blocked with PBS-Tween 20 (PBST, 0.05%, v/v) and BSA (1%, w/v, Sigma-Aldrich) during 30 min, and incubated with an in-house swine anti-ASFV serum conjugated with FITC. All procedures were performed at RT and all antibody incubations were performed in a dark humidified chamber to prevent fluorochrome bleaching. Vectashield mounting medium with DAPI (Vector Laboratories) was used to visualize the nucleus and the viral factories. Fluorescence images were acquired using a Leica DMIRE2 epifluorescence microscope and analysed with ImageJ open source software (version IJ 1.48g, National Institutes of Health, Bethesda, MD, USA) (Schneider, Rasband, & Eliceiri, 2012) and Adobe Photoshop CS5 software (Adobe Systems, Inc.).

4.2.6. Western blotting analysis

Mock-infected and ASFV-infected Vero cells, seeded in P6 multiwell plates, were washed twice with PBS and lysed in ice-cold modified RIPA buffer (25 mM Tris, pH 8.2, 150 mM NaCl, 0.5% NP40, 0.5% sodium deoxycolate, 0.1% SDS) supplemented with both a protease-inhibitor cocktail (cOmplete, Mini, EDTA-free from Roche) and a phosphatase-inhibitor cocktail (PhosStop, Roche). The whole-cell lysates harvested after 0, 1, 12, 24, 48 and 72 h of exposure to NaPB and at indicated time points post-infection, were further subjected to SDS-PAGE gel electrophoresis on 4–15% gradient polyacrylamide gel and transferred to a 0.2 µm

pore size nitrocellulose membrane (Whatman Schleider & Schuell) by electroblotting. Then, blot membranes were blocked with Tris-buffered saline/Tween-20 (TBS-T: 25mM Tris-HCl, pH 7.5, 150mM NaCl, 5% BSA [w/v], 0.05% Tween-20® [v/v]) during overnight, and incubated with the specific primary antibodies (1h, at RT). Afterwards, appropriate HRP-conjugated secondary antibodies were diluted and incubated during 1 hour at RT. All incubations were followed by three 10-min washes in TBS-T and protein detection was performed using a chemiluminescence detection kit (Pierce® ECL Western Blotting Substrate, Thermo Scientific), on Amersham Hyperfilm ECL (GE Healthcare). α–Tubulin expression was used as loading control.

4.2.7. Data analysis

Kruskal-Wallis Test was performed to compare the viral titers found in ASFV-infected cells treated with HDACi and in untreated infected cells (positive control). Differences were considered statistically significant for p values lower than 0.05 and results were presented as means ± standard errors of three independent experiments. IBM SPSS, version 23.0 for Windows, was the statistical software used (IBM Corp.).

4.3. Results

4.3.1. NaPB inhibits ASFV replication in a dose-dependent manner

In order to assess the antiviral activity of NaPB, VPA, TSA and SAHA, Vero cells were exposed to these drugs, before or during ASFV infection. The non-cytotoxic concentrations for each HDACi were previously determined (data not shown) and quite similar to the ones used in other mammalian cell lines (Huber et al., 2011; Mai et al., 2004; Mwakwari et al., 2010; Vázquez-Calvo et al., 2011). Interestingly, only NaPB fully inhibits the ASFV replication from 24 hpi onwards (Fig. 25A), in a dose-dependent manner (Fig. 25B), while VPA, TSA and SAHA only reduced half log of viral titers, at 24 and 48 hpi, around 90% (p=0.046, 0.043, respectively).

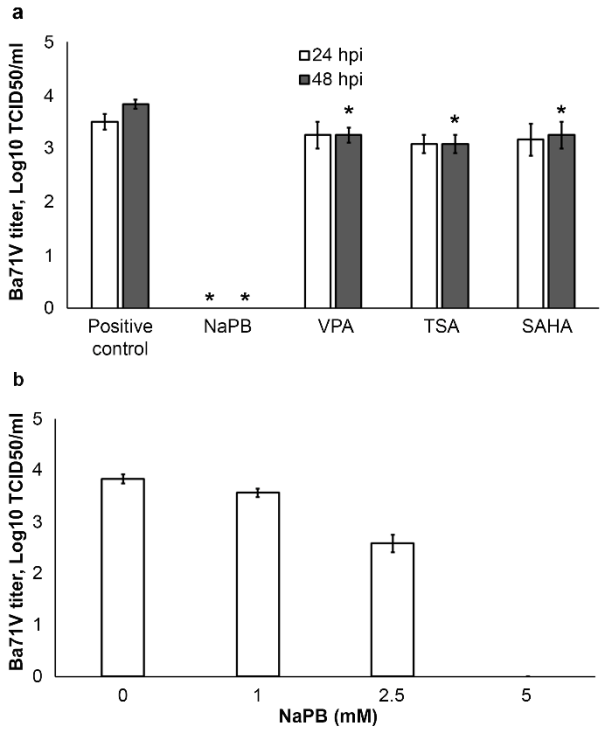

Figure 25. NaPB inhibits ASFV infection in a dose-dependent manner.
(a) HDACi treatments reduce viral progeny. Vero cells, either untreated or treated with NaPB (5 mM), VPA (25 mM), TSA (0.1 µM) or SAHA (1 µM), were infected with ASFV-Ba71V (MOI of 0.1). A complete inhibition of viral replication was observed at 24 and 48 hpi, contrasting with a reduction of 0.5 to 0.8 log found in cells exposed to other HDAC inhibitors. Asterisks represent a significant difference between the treated groups and the untreated control (p-value < 0.05). (b) Viral yields measured at 48 hpi, revealed that NaPB inhibits the ASFV infection in a dose-dependent manner, in cells exposed to NaPB 12 hours prior to infection (MOI of 0.1). Each column and error bars display the means ± standard error (SE) values. Data is representative of three independent experiments.

4.3.2. NaPB does not inactivate extracellular ASFV particles

To investigate whether the antiviral effects of NaPB are due to a disruption in the lipid composition of the ASFV envelope, as hypothesized for VPA (Vázquez-Calvo et al., 2011), ASFV-Ba71V particles were incubated with NaPB during 1 or 12 h, at 37ºC, before added to Vero cells. No significant differences were found in virus titers collected from Vero cells infected with viral particles exposed to NaPB and from supernatants of cells infected with untreated virus (data not shown), suggesting that NaPB does not affect the composition/infectivity of ASFV particles.

4.3.3. NaPB inhibits the ASFV late protein synthesis and disrupts the virus-induced H3K9/K14 hypoacetylation status

Knowing that NaPB does not inactivate the extracellular ASFV virions, inhibitory effects of this drug on viral protein expression were evaluated. A decrease in viral protein synthesis was detected in NaPB-treated cells, in particular, in intermediate-late viral proteins (Vp72 and Vp54, Fig. 26A), indicating that NaPB interferes with the late phase of ASFV infection. These results were supported by immunofluorescence studies, which showed that treated ASFV-infected

cells exhibit lower number (-81.02% at 24 hpi; 87.46% at 48 hpi) (data not shown) and smaller viral factories than untreated infected cells (Fig. 26B). Taking in consideration that ASFV modulates the epigenetic status of host cells and that HDAC inhibitors increase the acetylation levels of the histone H3 at lysine residues 9 and 14, we evaluated the acetylation status of this epigenetic marker during ASFV infection and investigated if NaPB changes the acetylation status of H3K9/K14 viral-induced. Immunoblot analysis revealed that ASFV promotes histone H3K9/K14 hypoacetylation and that NaPB disrupts this virus-induced modification (Fig. 26A), which may justify the antiviral effects of this drug.

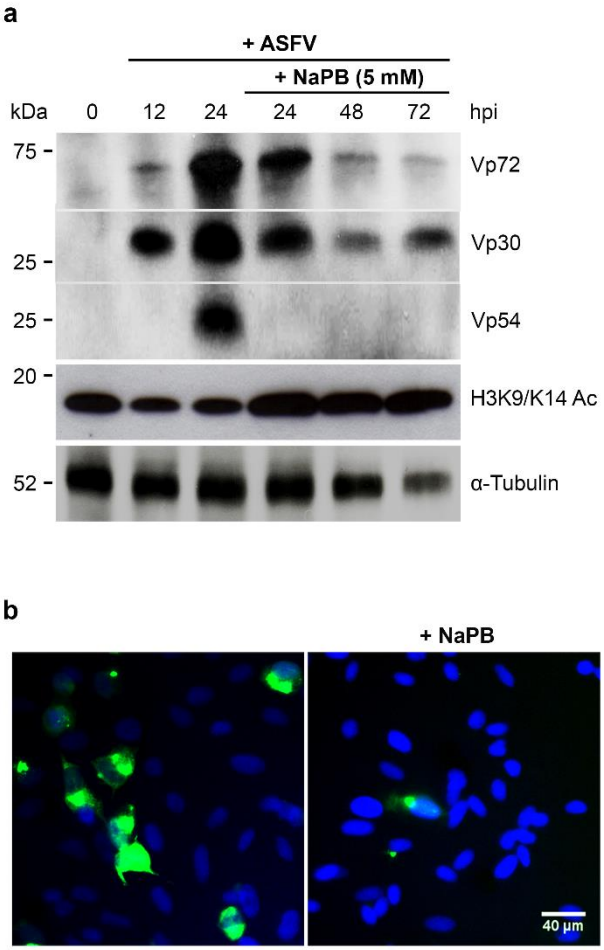

Figure 26. NaPB disrupts viral protein synthesis and alters the low acetylation levels of histone H3 at Lys9 and Lys14 induced by ASFV infection.
(a) Immunoblot analysis revealed that ASFV-infected cells, treated with NaPB, showed absence (Vp54) or lower levels of viral proteins (Vp30 and Vp72), at 24, 48 and 72 hpi, in comparison to untreated cells. In addition, ASFV-infected cells (12 and 24 hpi) showed low acetylation levels of H3K9/K14 when compared to mock cells, with infected cells treated with NaPB (5mM) displaying increased levels acetylation of H3K9/K14. α-tubulin was used as loading control. Molecular weights (kDa) of evaluated proteins are indicated on the left of immunoblot images. (b) NaPB treatment (5mM) reduces the number of infected cells and the size of the viral factories (MOI of 0.1, 48 hpi). An in-house produced swine anti-ASFV serum conjugated with FITC was used for viral protein detection.

4.3.4. NaPB and enrofloxacin act synergistically to abolish ASFV replication

Considering that in eukaryotic cells, HDAC inhibitors potentiate the DNA damage induced by topoisomerase II inhibitors (Guerrant et al., 2013; Marchion et al., 2004; Marchion et al., 2005b), we investigated if NaPB enhances the antiviral effects of the enrofloxacin, a ASFV-topoisomerase II poison (Freitas et al., 2016; Mottola et al., 2013). A complete inhibition of viral replication was observed when ASFV-infected cells were treated with half-protective concentration of NaPB (2.5mM) and enrofloxacin (25µg/ml), indicating that these drugs act synergically to inhibit ASFV replication. Indeed, although a small reduction of viral titers was detected when infected cells were treated with NaPB or enrofloxacin (-1.6 log and -2.2 log, respectively), no viral progeny was detected when both drugs were added to cells (Fig. 27).

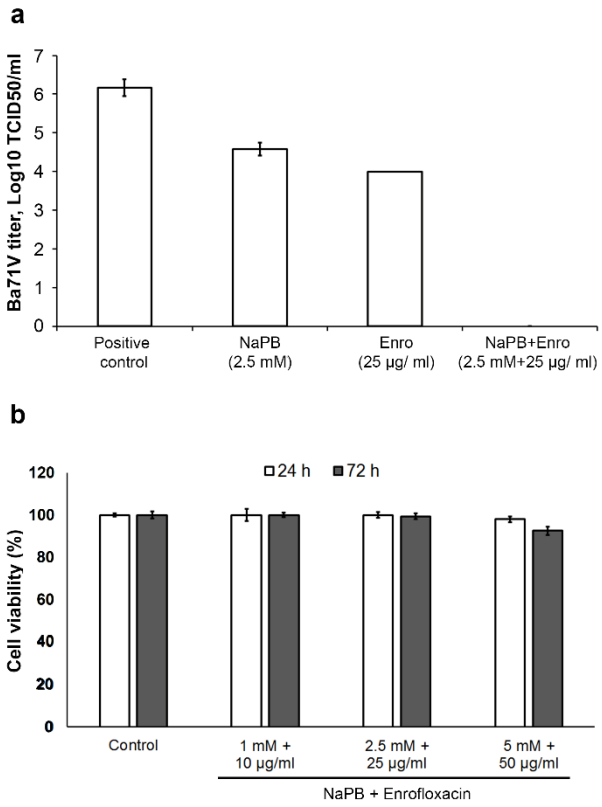

Figure 27. NaPB and enrofloxacin interact synergistically to inhibit ASFV infection.
A full protection against ASFV infection was achieved when infected cells (MOI of 0.1) were treated with NaPB (2.5mM) plus enrofloxacin (25µg/ml), 12 hours before and throughout infection, in contrast to ASFV-infected cells exposed to NaPB or enrofloxacin. Untreated cells infected with ASFV-Ba71V were used as positive control. Each column and error bars display the means ± standard error (SE) values and, data is representative of three independent experiments.

4.4. Discussion

Since HDACs are known to have a role in modulation of chromatin structure and in epigenetic regulation of gene expression, studies on the protective effects of HDACi have become a subject of intense interest, particularly in cancer and in virology, with multiple HDACi classes currently under investigation (Ahn et al., 2011; Archin et al., 2012; Bolden et al., 2006; Marks & Xu, 2009; Minucci & Pelicci, 2006; Placek & Berger, 2010).

In this study, we investigated the antiviral activities of four HDAC inhibitors. Remarkably, NaPB, a short fatty-acid HDACi, fully inhibits the ASFV replication at 24 and 48 hpi, contrasting with the other three HDAC inhibitors (VPA, TSA and SAHA), which only reduced the viral load between 0.5 and 0.8 log, at the same time points of infection. Interestingly, Vázquez-Calvo et al. (2011) have reported that VPA completely inhibits the release of ASFV progeny from 24 hpi onwards, using a higher VPA concentration (50 mM versus 25 mM), that showed cytotoxicity in our culture conditions. Similar to the impairment of viral protein synthesis caused by VPA in West Nile virus-infected cells (Vázquez-Calvo et al., 2011), NaPB also disrupts the ASFV protein production in Vero infected cells, in particular, the expression of intermediate/late viral genes.

Our results also showed that ASFV reduces the acetylation levels of histone H3 at lysine 9 and lysine 14, probably to silence critical genes (e.g. apoptosis or immune response-related), and granting extra time to successfully establish the infection, as reported for other viruses (e.g. human cytomegalovirus, HIV, hepatitis B virus, bovine leukaemia virus) (Jenke et al., 2014; Jiang, Espeseth, Hazuda, & Margolis, 2007; Merimi et al., 2007; Nevels, Paulus, & Shenk, 2004; Shestakova, Bandu, Doly, & Bonnefoy, 2001). Indeed, it is known that ASFV disrupts the epigenetic status of the host genome by recruiting HDAC2 and HP1 β/α into nucleus and increasing the trimethylation levels of histone H3 at lysines 9 and 27 (Simões et al., 2015a). Although the role of HDACs in ASFV infection is not fully understood (Bolden et al., 2006; Marks & Xu, 2009; Minucci & Pelicci, 2006), the inhibition of ASFV replication by NaPB, can be related with the disruption of the hypoacetylation status of H3 at Lys9 and Lys14 found in Vero-ASFV infected cells. Indeed, NaPB does not inactivate extracellular ASFV particles, contrary to the mechanism hypothesized for VPA, reinforcing the idea that its antiviral effects may derive from the genome-wide histone H3K9/K14 hyperacetylation, which may promote the expression of non-beneficial genes to infection progression, such as apoptosis-related genes and/or IFN-β (Shestakova et al., 2001). Moreover, the higher efficiency of NaPB to block ASFV infection may be due to the fact that this HDAC inhibitor shows selective activity against HDAC 2, 3 and 8, whereas TSA and SAHA mainly inhibit HDAC 3, 4, 6 and 7 and VPA has more efficiency against HDAC 3 and 8 (Blackwell, Norris, Suto, & Janzen, 2008). Taking in consideration that ASFV recruits HDAC 2 to viral factories (Simões et al., 2015a) and that only NaPB displays inhibitory activity against this HDAC enzyme, the modulation of this cellular enzyme seems to a strategy used by ASFV to successful replicates inside the cell. Our results

also revealed a synergistic interaction between NaPB and enrofloxacin, an ASFV-topoisomerase II poison (Freitas et al., 2016; Mottola et al., 2013), with half-effective concentrations being protective against ASFV. In cancer cells, this synergism has already been described (Bevins & Zimmer, 2005; Marchion et al., 2005a, 2005b), since HDACi promote histone hyperacetylation, enhancing the access and the trap of active topoisomerase II molecules by its poisons on DNA, leading to apoptosis (Cowell, Papageorgiou, Padget, Watters, & Austin, 2011; Marchion et al., 2005).

In conclusion, our results strongly suggest that ASFV interacts with cellular HDACs to modulate the epigenetic status of host cells, inducing a H3K9/K14 hypoacetylation status. As reported for other viruses (Chiocca et al., 2002; Punga & Akusjärvi, 2000; Valls et al., 2007), this epigenetic modification seems to be used by ASFV to evade the host's defence systems and to establish a successful infection. We also demonstrated that NaPB promotes histone H3K9/K14 hyperacetylation, an epigenetic status associated with active gene expression. Although further studies are needed, HDACs inhibitors may be used to control ASFV infection in vivo, combined or not with ASFV-Topoisomerase II inhibitors, to reinforce the implementation of required sanitary measures for the control of the disease in occurring outbreaks.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

No animals were used for the work presented in this manuscript.

CHAPTER V

General Discussion, Conclusions and Future Directions

African swine fever virus is a complex virus whose genome encodes approximately 150 proteins, some of them well characterized and involved in transcription, replication or required for virion assembly (Dixon et al., 2013). However, and despite all the scientific research efforts developed over the last decades, the biological function of numerous viral proteins remains uncharacterized. These gaps of knowledge together with the need of a better understanding on ASFV biology and viral-host interactions, contribute to the failure in the development of an effective vaccine against ASF (Rock, 2016). In this perspective, this work aimed at characterizing the ASFV ORF A104R, previously described as putatively coding for a protein with significant sequence homology with bacterial histone-like protein (Borca et al., 1996; Neilan et al., 1993). Despite of having been identified over two decades ago and described as the first histone-like protein encoded by an eukaryotic virus, the functional and biological relevance of pA104R remain to be explained.

To contribute for the characterization of ASFV ORF A104R, this work aimed at elucidating the role of this protein in ASFV infection, thus improving the knowledge on ASFV biology and giving support to a further use of pA104R as a possible target for vaccine design.

5.1. The role of pA104R in ASFV infection and modification of histone acetylation during ASFV infection

The electrophoretic mobility shift assay (EMSA) was used to characterize the DNA-binding activity of ASFV-pA104R. In agreement with some bacterial histone-like proteins and other viral DNA-binding proteins, our results showed that this protein binds to both single- and double-stranded DNA, as suggested by Borca et al. (1996), though having higher affinity to ds-DNA, with a DNA-binding size of approximately 14-16 nt. Interestingly, these results are in line with others obtained in studies using bacterial histone-like proteins and other viral DNA-binding proteins (Balandina, Kamashev, & Rouviere-Yaniv, 2002; Kamashev, Balandina, Mazur, Arimondo, & Rouviere-Yaniv, 2008; Loregian et al., 2007; Rochester & Traktman, 1998). Concerning the DNA-binding properties, the maintenance of pA104R activity in extreme environmental conditions (time of incubation, pH, temperatures) is remarkable, and may sustain the complex ASFV infectious cycle in different epidemiologic scenarios. These *in vitro* results suggest that pA104R may remain active in soft tick vectors (*Ornithodoros spp.*), being of major interest the development of further research towards the role of ASFV-pA104R in the viral infection of this vector.

Two of the three aminoacid residues identified by Borca et al. (1996) as important for DNA-binding were experimentally evaluated. While the mutation of proline at position 74 to alanine did not change the enzymatic activity, the mutation of the arginine at position 69 to alanine reduced it approximately 3-fold. Additionally, the results obtained by using the site-directed mutagenesis also suggest that the positive charge of this residue seems to be responsible for the DNA-binding activity. The identification of the catalytic site and domains could contribute

to better characterize the pA104R-DNA binding. Using crystallography, it will be possible to identify a core region within the pA104R-DNA interface that could be targeted with structure-based inhibitors, turning this viral protein a potential target for the development of therapies against ASF.

Regardless of the *in vitro* studies towards pA104R activity, the precise role of this protein in ASFV infection remains unclear. The analysis of the transcription pattern of ORF A104R showed that transcripts of this gene are detectable during an intermediate to late phase of infection. These findings were corroborated by immunoblot analysis of whole-cell extracts collected at different time-points of infection and are in agreement with others previously obtained (Borca et al., 1996), suggesting that this protein may be involved in the viral transcription and replication during the cytoplasmic phase of infection. The significant sequence homology and structural similarities to bacterial histone-like proteins observed for the first time by Borca et al. (1996) and Neilan et al. (1993), also prompts the idea that the viral histone-like protein may be responsible for wrapping virus genome within the virion. In bacteria, these proteins are known to induce DNA topological changes such as supercoiling, maintaining DNA architecture and also play an important role in the regulation of gene expression (Dorman, 2013; Dorman & Deighan, 2003; Macvanin & Adhya, 2012; Oberto et al., 2009). In virology field, DNA viruses, as their hosts, face the challenges of neutralizing the negative charge of DNA and condensing their genomes inside the host cells and viral particles. Although the mechanisms used by viruses to overcome this challenge are not fully understood, some studies suggest that some viruses encode their own histone-like protein, while others compact their genomes with host core histones (Lieberman, 2006; Lu & Triezenberg, 2010). For example, whereas adenoviruses encode adenoviral core proteins which promote genome condensation, some small DNA tumour viruses, such as polyomavirus and simian virus 40 (SV40), use cellular histones during virion encapsidation (Giberson, Davidson, & Parks, 2012; Lieberman, 2006). On the other hand, herpesvirus contains no protein associated with DNA within the capsid, even though its genome is assembled into nucleosomal structures during latency and early stages of lytic infection (Kent et al., 2004; Lu & Triezenberg, 2010).

The limited coding capacity of most viruses usually restricts the incorporation of some enzymes dedicated exclusively to chromatin functions. This does not seem to be the case of ASFV, since its large DNA genome encodes a wide range of enzymes (e.g. histone-like protein, type II topoisomerase, DNA helicases) that could allow the self-replication, theoretically without needing the host DNA replication machinery. However, the large length of the ASFV genome (170-190 kbp) may difficult the viral DNA packaging before encapsidation and this may justify why this notable virus encodes for a histone-like protein. Other members of the NucleoCytoplasmic Large DNA Viruses (NCLDV) cluster such as lausannevirus and marseillevirus also encode for proteins with predicted DNA-binding activity, which present similarities to eukaryotic and archaeal histones (Thomas et al., 2011). Indeed, the packaging

of the ASFV genome seems to be a challenging step in the nucleoid morphogenesis since viral particles without DNA have been shown to be frequently released from infected cells (Salas & Andrés, 2013). These findings may also suggest that this event is very complex, and different factors could regulate the viral DNA compaction.

Although a knockout mutant on A104R gene would be needed to fully understand the exact role of this protein on ASFV infection, it is our belief that the supercoiling assay developed in this work contributes to clarify this question. The DNA supercoiling activity observed when pA104R and the recently characterized ASFV-topoisomerase II (Coelho et al., 2016, 2015; Freitas et al., 2016; Garcia-Beato et al., 1992b) were added to a relaxed DNA plasmid, reinforces the idea that the ASFV genome encodes for proteins involved in nucleoid formation, namely in viral DNA condensation.

Furthermore, the immunofluorescence analysis of pA104R cellular localization performed in this study contributes to better characterize the role of pA104R on host cell infection. While the co-localization with the viral factories corroborate the involvement of this protein in DNA-related mechanisms, its presence in cell nucleus suggests that pA104R may participate in other biological events. It may bind and interact with host cell DNA, manipulating the host gene expression on its benefit. Viral proteins described in other viruses have profound effects on chromatin structure and formation. The presence of histone-like sequences (histone mimics) in some viral proteins may contribute to the remarkable ability of viruses to compete with the cognate histone sequences for their common binding partners (Schaefer, Ho, Prinjha, & Tarakhovsky, 2013). Moreover, several viral proteins have been found to interact with cellular chromatin-modifying enzymes such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), affecting post-translational modification of host histones and non-histone proteins (Chakravarti et al., 1999; Chiocca et al., 2002; Lomonte et al., 2004; Punga & Akusjärvi, 2000; Valls et al., 2003; Zhang & Jones, 2001). These mechanisms interfere with the establishment of transcriptional complexes and gene expression, including those that play an important role in regulation of host antiviral response, and may represent an evading host response mechanism.

Although no scientific evidences until now support the activity mechanisms of ASFV-pA104R, the ASFV-induced hypoacetylation status observed in this study suggests that ASFV can modulate the host-cell epigenetic and consequently gene expression. This hypothesis is also supported by a study developed by Simões et al. (2015a) where the heterochromatinization of the host genome and the redistribution of HDAC2, trimethylated lysine-9 of histone H3 (H3K9me3), and HP1β was reported in infected cells. The modification of these epigenetic marks along with the H3K9K14 hypoacetylation found in this work are classically correlated with gene silencing in eukaryotic cells and may indicate that ASFV subverts different cellular mechanisms, controlling the access of transcription machinery to host genes, namely those related to cellular anti-viral response.

Further studies identifying the exact mechanism and/ or the viral genes responsible for the modification of host epigenetic signatures during infection will contribute to better understand the ASFV biology and the virus-host interactions, and will lead to new opportunities for testing new promising molecular targets as antiviral therapies.

5.2. HDACi as potential drugs for the study of viral-host interaction and for the development of antiviral agents

In this study, the *in vitro* effect of four HDAC inhibitors (NaPB, VPA, TSA and SAHA) on ASFV infection were evaluated as a way to study the importance of cellular HDACs in infection as well as to screen if these inhibitors are potential antiviral drugs. Our work demonstrates that NaPB inhibits completely ASFV infection in Vero cells, reinforcing the above-described hypothesis that ASFV can interact with host HDACs in order to regulate host histones acetylation on its benefit, since it was proven that this inhibitor also disrupts the virus-induced H3K9K14 hypoacetylation. These findings are schematically illustrated in the proposed working model for the antiviral mechanism of NaPB, presented in Figure 28. Additionally, as NaPB has higher efficiency to inhibit HDAC2 than any of other HDACi used (Blackwell et al., 2008), the superior antiviral effects of this drug as compared to VPA, TSA and SAHA, suggest that the recruitment of HDAC2, observed on a previous study (Simões et al., 2015a), constitutes an important strategy used by ASFV to establish a successful replication in the host cells. Nevertheless, the interaction of ASFV with cellular HDACs is still unclear and further studies addressing this subject should clarify this host-viral interplay.

Knockdown of specific HDACs in infected cells will be important to confirm the viral-host interaction suggested in this work and will identify which cellular HDACs are usurped by ASFV. This information will be crucial to choose more selective HDACi recently developed.

As the inhibition of histone deacetylation has been previously correlated with the expression of non-beneficial genes to other viral infection progression, such as apoptosis-related genes and/or IFN-β (Shestakova et al., 2001), the identification of the host genes that have their expression altered in cells treated with NaPB, will also be important to better understand the antiviral mechanism of this drug.

Since effective prophylactic and therapeutic measures are lacking against ASFV, the identification of novel viral protein-host interactions may open new insights for the development of antiviral therapies. An inhibitor that successfully acts on ASFV infectious cycle and/ or targets host cell factors involved in virus replication, may be a useful tool to avoid the spread of the ASFV in naturally occurring outbreak areas, granting time to the application of the required sanitary measures (Zakaryan & Revilla, 2016). Moreover, a rational design of efficient vaccines may be established if future studies determine which ASFV genes could interact with HDACs.

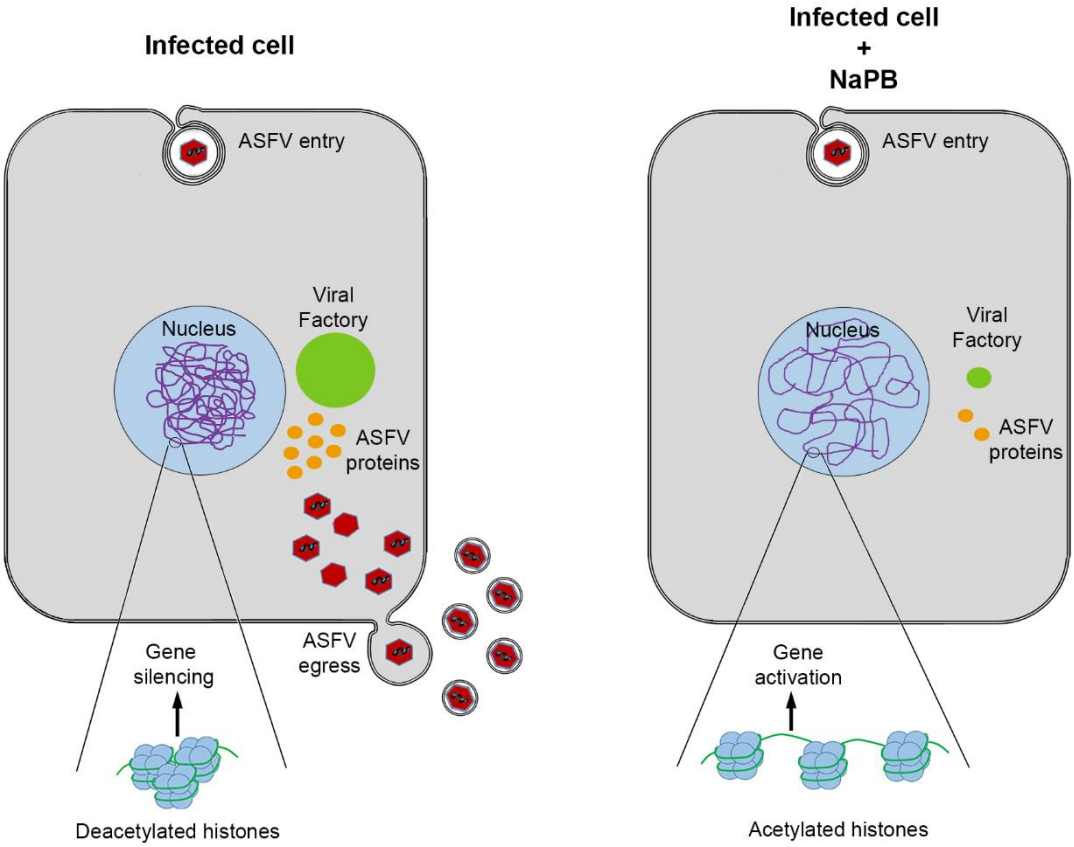

Figure 28. Proposed working model for the antiviral activity of NaPB on ASFV-infected cells. During infection, the ASFV promotes heterochromatinization of the host nucleus by inducing H3K9/K14 hypoacetylation, thus repressing transcription of host genes. By inhibiting HDACs, NaPB promotes an open chromatin conformation, facilitating the expression of non-beneficial genes for infection (e.g. apoptosis-related genes and IFN-β).

5.3. The development of a DISC vaccine targeting pA104R

Currently, an effective vaccine against ASF is not available. This reality became even more relevant after the recent introduction and dissemination of the virus in Eastern Europe, turning ASF a continuous threat to pig husbandry. At these countries and others affected the only control measures available are based on rapid laboratory detection and diagnosis and implementation of sanitary/biosecurity measures, including animal slaughter, restriction of animal movements and surveillance (Wieland et al., 2011).

Nevertheless, an ASF vaccine could indeed be achieved as surviving infected pigs develop resistance to infection with the homologous virus (Boinas et al., 2004; Hamdy & Dardiri, 1984; King et al., 2011; Leitão et al., 2001; Mebus & Dardiri, 1980). Development of vaccines against ASF carried out over the past decades has been mostly based on virus inactivation and attenuation. While inactivated vaccines have failed to induce protection (Forman et al., 1982; Mebus, 1988; Stone & Hess, 1967), a long-term resistance to homologous virus challenge was observed in pigs infected with live-attenuated or naturally occurring low virulent ASFV isolates (King et al., 2011; Lacasta et al., 2015; Leitão et al., 2001; Lewis et al., 2000; Mulumba-Mfumu, Goatley, Saegerman, Takamatsu, & Dixon, 2016; O'Donnell et al., 2015; Reis et al., 2016).

However, the live-attenuated ASF vaccines, obtained either by cell cultures passages or by targeted deletions, have failed to confer protection against heterologous isolates and have also shown significant safety issues unacceptable for vaccine use.

Further studies addressing ASFV infection, with the goal to identify ASFV genes whose deletion may reduce virulence and increase the host immune response, are mandatory to overcome these difficulties. The failure in obtaining a protective vaccine indeed emphasizes the need of an improvement in the knowledge about ASFV biology and opens a new window of opportunities to develop a vaccine against ASF. In this scenario, a replication-defective or a defective infectious single-cycle (DISC) viral particle emerges as a refreshing and promising approach for immunization against ASFV once the classical approaches have not been successful.

These mutant viruses are defective for one or more functions, essential for virus replication, and combine some of the advantages of both classical types of viral vaccines, by expressing viral antigens that can lead to a strong immune response, and simultaneously being safe (Dudek & Knipe, 2006). The replication-defective mutant viruses are generated by deleting essential early genes, resulting in some viral gene expression but without progeny virus production (Dudek & Knipe, 2006). On the other hand, DISC viruses are defective in a protein involved in late viral mechanisms, leading to a normal replication cycle and viral progeny production. However, these virions are non-infectious so the infection does not spread to a second round of cells (Crosby et al., 2015; Crosby et al., 2017; Dudek & Knipe, 2006; Murakami, Terasaki, Ramirez, Morrill, & Makino, 2014).

Taking into consideration initial results obtained in this work, it is expected that a deletion mutant in A104R gene will replicate its genome in the host cells but will not be able to package it within the virion, resulting on “empty” non-infectious virion that will be unable to initiate a second cycle of infection. This idea was reinforced by results obtained in the siRNA experiments showing a reduction of viral progeny production when the A104R gene was downregulated. Additionally, since the reduction of mRNA of A104R caused a decrease of late gene (B646L) transcription, not influencing early gene transcription (CP204L), it is plausible that an ASFV deletion mutant on A104R gene can be a DISC mutant.

Expecting that a deletion mutant on the A104R gene would be a DISC mutant, a helper cell-line expressing this viral gene was developed to obtain and propagate the recombinant ΔA104R virus. A Vero cell-line expressing pA104R was indeed successfully obtained in this study, however the pA104R levels are much lower comparing to ASFV-Ba71V infected Vero cells. This finding together with our failed attempts to isolate and amplify the recombinant ASF virus, suggests that this complementing Vero cell-line does not support the replication of recombinant ΔA104R virus.

On the other hand, the strategy developed to construct the recombination vector and obtain the recombinant ΔA104R virus by homologous recombination seems to be a successful

approach since the recombinant viruses expressing the reported GUS gene were identified by the presence of blue plaques when X-Gluc was added.

To our knowledge, this is the first study aiming a generation of a DISC ASFV particle and, despite the isolation of the recombinant virus was not well succeed, we believe that the strategy used will be employed in future works. The main problem in the generation of virus lacking essential genes, is the production of helper cell-line expressing the viral gene deleted, thus an exhaustive research on the development of this cell line is crucial to obtain a replication defective or a DISC ASFV. Further studies on the different porcine monocyte-derived cell line (e.g. ZMAC, IPAM WT, IPAMCD163, WSL and CΔ2+) (Calzada-Nova, Husmann, Schnitzlein, & Zuckermann, 2012; Chitko-McKown et al., 2013; Cong et al., 2013; Keil, Giesow, & Portugal, 2014) and other methods to obtain complementary cell lines (e.g. CRISPR/Cas9) (Cong et al., 2013) may solve the problems found in this work in order to develop a safe and protective DISC vaccine against ASF.

5.4. Concluding remarks

The recent alarming spread of ASF in Eastern Europe demands immediate countermeasures, with the development of a vaccine as a high priority. For this purpose, an intensive research on ASFV biology and on viral-host interactions is mandatory. The characterization of pA104R DNA-binding activity and the study of the virus-host interactions outlined in this study are fundamental to better understand ASFV biology and may be used to develop potential therapeutic strategies for ASF, simultaneously opening new insights for vaccine development against the disease.

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